# EXHIBIT A

# ENZYME NOMENCLATURE
# 1992


## RECOMMENDATIONS OF THE NOMENCLATURE COMMITTEE OF THE INTERNATIONAL UNION OF BIOCHEMISTRY AND MOLECULAR BIOLOGY ON THE NOMENCLATURE AND CLASSIFICATION OF ENZYMES

This edition is a revision of the Recommendations (1984) of the Nomenclature Committee of IUB, and has been approved for publication by the Executive Committee of the International Union of Biochemistry and Molecular Biology

Prepared for NC-IUBMB by Edwin C. Webb


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1.1.3.3 Malate oxidase
REACTION: *(S)*-Malate + $O_2$ = oxaloacetate + $H_2O_2$
SYSTEMATIC NAME: *(S)*-Malate:oxygen oxidoreductase
COMMENTS: A flavoprotein (FAD)
REFERENCES: 847, 3522

1.1.3.4 Glucose oxidase
REACTION: β-D-Glucose + $O_2$ = D-glucono-1,5-lactone + $H_2O_2$
OTHER NAME(S): Glucose oxyhydrase
SYSTEMATIC NAME: β-D-Glucose:oxygen 1-oxidoreductase
COMMENTS: A flavoprotein (FAD)
REFERENCES: 391, 903, 2459, 2460

1.1.3.5 Hexose oxidase
REACTION: β-D-Glucose + $O_2$ = D-glucono-1,5-lactone + $H_2O_2$
SYSTEMATIC NAME: D-Hexose:oxygen 1-oxidoreductase
COMMENTS: A copper glycoprotein. Also oxidizes D-galactose, D-mannose, maltose, lactose and cellobiose
REFERENCES: 360, 361, 4817

1.1.3.6 Cholesterol oxidase
REACTION: Cholesterol + $O_2$ = cholest-4-en-3-one + $H_2O_2$
SYSTEMATIC NAME: Cholesterol:oxygen oxidoreductase
REFERENCES: 4111, 4689

1.1.3.7 Aryl-alcohol oxidase
REACTION: An aromatic primary alcohol + $O_2$ = an aromatic aldehyde + $H_2O_2$
SYSTEMATIC NAME: Aryl-alcohol:oxygen oxidoreductase
COMMENTS: Oxidizes many primary alcohols containing an aromatic ring; best substrates are (2-naphthyl)methanol and 3-methoxybenzyl alcohol
REFERENCES: 1312

1.1.3.8 L-Gulonolactone oxidase
REACTION: L-Gulono-1,4-lactone + $O_2$ = L-*xylo*-hexulonolactone + $H_2O$
SYSTEMATIC NAME: L-Gulono-1,4-lactone:oxygen 2-oxidoreductase
COMMENTS: A flavoprotein (FAD). The product spontaneously isomerizes to L-ascorbate
REFERENCES: 2211, 2546

1.1.3.9 Galactose oxidase
REACTION: D-Galactose + $O_2$ = D-*galacto*-hexodialdose + $H_2O_2$
SYSTEMATIC NAME: D-Galactose:oxygen 6-oxidoreductase
COMMENTS: A copper protein
REFERENCES: 203

1.1.3.10 Pyranose oxidase
REACTION: D-Glucose + $O_2$ = 2-dehydro-D-glucose + $H_2O_2$
OTHER NAME(S): Glucose 2-oxidase
SYSTEMATIC NAME: Pyranose:oxygen 2-oxidoreductase
COMMENTS: A flavoprotein (FAD). Also oxidizes D-xylose, L-sorbose and D-glucono-1,5-lactone, which have the same ring conformation and configuration at C-2, C-3 and C-4
REFERENCES: 2280, 3003, 3545, 4220

# EXHIBIT B

JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

# Analysis and Modeling of the Ferulic Acid Oxidation by a Glucose Oxidase−Peroxidase Association. Comparison with a Hexose Oxidase−Peroxidase Association

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A commercial glucose oxidase (GOX) from *Aspergillus niger* was partially characterized. The enzyme exhibited a two-step transfer mechanism, and the kinetic constants toward glucose and oxygen were determined. Under conditions similar to dough making (glucose concentration and pH), GOX does not exhibit maximum activity. A hexose oxidase (HOX) from *Chondrus crispus* was partially characterized as well. The HOX activity is not far from the optimum in the kneading conditions (pH and glucose concentration). A peroxidase (POD) purified from wheat germ was used to oxidize ferulic acid in the presence of GOX or HOX. Hydrogen peroxide produced during the glucose oxidation activates the wheat germ POD. Ferulic acid oxidation in solutions containing different ratios of POD + GOX or HOX + POD was followed by UV spectrophotometry. For the same dosage, the HOX−POD system is the most efficient for peroxidase activation. Using absorbance data and kinetic constants of GOX and POD, a mathematical model describing the release or consumption of the different reactants (hydrogen peroxide, oxygen, and ferulic acid) in the medium was developed, and experimental data correlated well with calculated values. The results obtained will be applied to investigate the effect of GOX and HOX activities on the rheological properties of dough.

KEYWORDS: Ferulic acid; peroxidase; bread making; glucose oxidase

## INTRODUCTION

Potassium bromate has been used extensively, as an oxidant, to improve the quality of flour during dough making. It enhances the baking performance and aids the development of a strong relaxed gluten matrix (*1*). In the last two decades, several toxicological studies have shown that potassium bromate causes tumors in rats (*2*), and a few cases of poisoning have been reported (*3*). As a consequence, in 1987, the International Cancer Research Agency classified potassium bromate as a carcinogen (*4*). Baking industry researchers have endeavored to identify substitutes for potassium bromate, and one of the possibilities is the use of enzymes. Additions of glucose oxidase (GOX) (EC 1.1.3.4), hexose oxidase (HOX) (EC 1.1.3.5.) or phytase (*4*−*7*) have been suggested during dough mixing. The use of HOX (*5*) or GOX, alone (*8*, *9*), or in combination with hemicellulase (*10*) or α-amylase (*11*) has also been proposed to improve baking performance.

GOX, in the presence of molecular oxygen, catalyzes the oxidation of glucose to gluconolactone and hydrogen peroxide (*12*). GOX kinetics are Michaelian type with a Ping-Pong mechanism. *Aspergillus niger* GOX has been the most studied (*5*, *13*, *14*), and its effect on bread making have been extensively reported. Faisy and Neyreneuf (*10*) proposed the use of a GOX/hemicellulase association as a substitute of potassium bromate. They reported that the addition of both enzymes increased bread volume. Vemulapalli et al. (*15*, *16*) compared the use of chemical oxidants such as calcium peroxide or potassium bromate with GOX in bread making and found that doughs where GOX is added are strong and dry. Miller and Hoseney (*17*) reported similar results. Ameille et al. (*18*) also suggested an activation of endogenous peroxidase (POD) (EC 1.11.1.7) by GOX addition in dough. An oxidative gelation of water-soluble pentosans catalyzed by POD, and causing a limitation in water mobility could explain these effects. The ability of POD, in the presence of hydrogen peroxide, to promote gelation of pentosans (via ferulic acid) is well documented (*19*−*23*). Figueroa-Espinoza and Rouau (*24*) and later Figueroa-Espinoza et al. (*25*) confirmed that the cross-linking of pentosans by a fungal laccase, a horseradish peroxidase (HRP), or manganese peroxidase takes place through the coupling of their esterified ferulic acid. In a previous communication, we described the oxidation of ferulic acid and 5-*O*-(*trans*-feruloyl)-L-arabinose by a wheat germ POD (*26*) It was shown that ferulic acid dimers can be formed enzymatically and assumed that the enzyme can use the ferulic acid esterified to arabinoxylans as substrate, leading to the formation of a gel. These results are consistent with those of Figueroa-Espinoza et al. (*25*).

HOX, unlike GOX can use several monosaccharides and oligosaccharides as substrates and it catalyses the conversion

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of the latters into corresponding lactones with the formation of hydrogen peroxide. Poulsen and Bak Hostrup (*5*) compared the effects of HOX and GOX in dough and bread. They showed that HOX caused dough strength and increased bread volume more efficiently than GOX in the same dosage.

The effect of GOX on sulfhydryl (SH) groups has also been investigated (*5*, *15*). SH groups of gluten protein are oxidized to disulfide bridges (S–S) when GOX is added, resulting in a dough strengthening and a better gas retention during fermentation (leading to bread with increased volume). Furthermore, it was confirmed that GOX is able to activate endogenous POD. Kieffer et al. (*27*) reported that the addition of HRP, $H_2O_2$, and catechol improved the rheological properties of wheat dough. This effect was attributed to the oxidation of protein-bound cysteine to cystine. The addition of soya POD had the same effect (*28*, *29*). Labat et al. (*30*) investigated the effect of laccase and ferulic acid addition on wheat flour doughs and showed that the addition of both compounds caused an increase in the oxidation of SH groups and the rate of protein depolymerization during mixing. A coupling reaction involving enzymatically (laccase) generated feruloyl radicals and thiol radicals generated through the mechanical breakdown of interchain disulfide bounds could explain their findings.

GOX supplementation leads to changes in the structural properties of the dough affecting its consistency and enhancing the bread volume (*5*, *9*, *16*, *31*).

Addition of GOX or HOX may also modify the balance between the different enzymatic systems present in dough. Hydrogen peroxide production could activate catalase and peroxidase systems and oxygen consumption could reduce lipoxygenase activity. Rakotozafy et al. (*32*) reported that the loss in lipoxygenase activity, usually observed during mixing, is reduced when GOX is added. According to these authors, the decrease in oxygen available could limit the lipoxygenase activity and therefore its catalytic denaturation during mixing.

In this paper, the oxidation of ferulic acid by a GOX–POD association or by a HOX–POD association (hydrogen peroxide enzymatically supplied) is investigated to understand the effects of GOX and HOX in bread making. GOX and HOX were kinetically characterized, to define the optimum conditions for the enzyme activity. The effect of altering GOX/POD relative concentrations was analyzed by spectrophotometry. A mathematical model, which describes the release or consumption of the different reactants in the medium, was developed and a comparison was made with the experimental data.

HOX was partially characterized and the ability of the HOX–POD system to oxidize ferulic acid was compared with the ability of the GOX–POD system to do so.

## MATERIALS AND METHODS

**Plant Material.** Industrial germ was provided by Les Moulins Soufflet (Nogent-sur-Seine, France) and defatted by cold acetone according to Nicolas et al. (*33*).

**Enzymes.** Wheat germ POD was purified according to Billaud et al. (*34*) and the major cationic fraction was used for the study. GOX was extracted from *Aspergillus niger*; its activity was 760 nkat/mg in the standard assay conditions. HOX, extracted and purified from the red algae *Chondrus crispus*, was a gift from Danisco Ingredients (Brabrand, Denmark), its activity was 33 nkat/mL in the standard assay conditions.

**Chemicals.** Hydrogen peroxide 30% (v/v), *m*- and *o*- phosphoric acids, D-glucose, calcium chloride, and ascorbic acid were purchased from VWR (Paris, France). Ferulic acid (FA) was purchased from Sigma Chemical (St. Louis).

Table 1. Apparent Kinetic Constant Values of GOX for $[O_2] = 220$ $\mu$M

| | pH 5.0 | pH 5.6 | pH 6.0 |
|---|---|---|---|
| $K_{mappG}^{GOX}$ (mM) | 57.6 | 22 | 16 |
| $V_{mappG}^{GOX}$ (nkat/mg) | 1020 | 1130 | 580 |
| $V_{mappG}^{GOX}/K_{mappG}^{GOX}$ | 17.7 | 51.4 | 36.3 |

**Peroxidase Assay.** The POD activity was determined as previously described (*26*), and the spectrophotometric data were converted into residual ferulic acid concentrations ($[FA]_{residual}$) using the relationship formerly established (*26*)

$$[FA]_{residual}(\mu M) = [FA]_0 + 18.7(Abs_{\infty} - Abs_t)^2 - 119(Abs_{\infty} - Abs_t)$$

where $[FA]_0$ and $(Abs_\infty - Abs_t)$ represent the initial ferulic acid concentration ($\mu$M) and the decrease in absorbance value at 310 nm during the reaction time $t$, respectively. POD activity is expressed in nanokatals (nmol of FA consumed per second). In these assay conditions, the purified wheat germ POD used in this study had an activity of 0.26 $\mu$kat/mL and the slope of the absorbance decrease remained proportional to the enzyme amount until an activity of 10 nkat in the reaction solution.

**Glucose Oxidase Assay.** The GOX activity was determined polarographically using glucose (220 mM) dissolved in a 100 mM acetate buffer pH 5.6 saturated by air at 30 °C according to Rakotozafy et al. (*32*). Activity is expressed in nanokatals (nmol of oxygen consumed per second).

**Hexose Oxidase Assay.** The HOX activity was determined polarographically using glucose (50 mM) dissolved in a 100 mM acetate buffer solution at pH 5.6 saturated with air at 30 °C. Activity is expressed in nkat (nmol of oxygen consumed per second in the assay conditions).

**GOX–POD Mixtures.** Evolution of FA in GOX-POD mixtures was analyzed by UV-spectrophotometry (310 nm) according to Garcia et al. (*26*). GOX solution used in these experiments was prepared at a concentration of 0.27 mg/mL. Wheat POD used in these experiments had a $V_m^{POD}$ value of 5.4 $\mu$kat/mL of enzymatic solution (corresponding to the 0.26 $\mu$kat/mL determined in the standard assay conditions).

The composition of mixtures containing a fixed GOX amount was as follows: 6.7 $\mu$g of GOX (5.1 nkat), 90 $\mu$M FA, 50 mM D-glucose, 10–100 $\mu$L of POD (2.6–26 nkat), and 20 mM $CaCl_2$ in 100 mM acetate buffer pH 5.6 (3 mL final volume).

The composition of mixtures containing a fixed POD amount was as follows: 25 $\mu$L of POD (6.5 nkat), 90 $\mu$M FA, 50 mM D-glucose, 2.7–27 $\mu$g GOX (2.05–20.5 nkat), and 20 mM $CaCl_2$ in 100 mM acetate buffer pH 5.6 (3 mL final volume).

The composition of mixtures for the analysis of the ascorbic acid effect was as follows: 80 $\mu$L of POD (21 nkat), 27 $\mu$g of GOX (20.5 nkat), 90 $\mu$M FA, 20 mM $CaCl_2$, 50 mM D-glucose, and 100 mM acetate buffer pH 5.6 containing from 0 to 500 $\mu$M ascorbic acid (3 mL final volume). Evolution of FA in GOX–POD mixtures containing ascorbic acid was followed both by UV-spectrophotometry and ECD-HPLC, according to Garcia et al. (*26*).

**Comparison of GOX–POD and HOX–POD Mixtures.** Evolution of FA in the mixtures was followed by spectrophotometry according to Garcia et al. (*26*). The composition of the mixtures is as follows: 25 $\mu$L of POD (6.5 nkat), 90 $\mu$M FA, D-glucose (from 2 to 50 mM), and 0.42 to 2.6 nkat of GOX or HOX in 100 mM acetate buffer pH 5.6 (3 mL final volume).

## RESULTS AND DISCUSSION

**Glucose Oxidase Characterization.** The effect of glucose concentration on the enzyme activity was determined at three different pHs, at one oxygen concentration (220 $\mu$M). The enzyme affinity toward glucose increases with pH. Conversely, $V_{mappG}^{GOX}$ values decrease when pH increases (Table 1). The best efficiency ($V_{mappG}^{GOX}/K_{mappG}^{GOX}$) of the enzyme was found at pH 5.6

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**Chemicals.** Hydrogen peroxide 30% (v/v) *m*- and *o*- phosphoric acids, D-glucose, calcium chloride, and ascorbic acid were purchased from VWR (Paris France) Ferulic acid (FA) was purchased from Sigma Chemical (St Louis)

Table 1. Apparent Kinetic Constant Values of GOX for $[O_2] = 220$ $\mu$M

| | pH 5 0 | pH 5 6 | pH 6.0 |
|---|---|---|---|
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$$[FA]_{residual}(\mu M) = [FA]_{t0} + 18\,7(Abs_{t0} - Abs_t)^2 - 119(Abs_{t0} - Abs_t)$$

where $[FA]_{t0}$ and $(Abs_{t0} - Abs_t)$ represent the initial ferulic acid concentration ($\mu$M) and the decrease in absorbance value at 310 nm during the reaction time $t$, respectively. POD activity is expressed in nanokatals (nmol of FA consumed per second) In these assay conditions, the purified wheat germ POD used in this study had an activity of 0 26 $\mu$kat/mL and the slope of the absorbance decrease remained proportional to the enzyme amount until an activity of 10 nkat in the reaction solution

**Glucose Oxidase Assay.** The GOX activity was determined polarographically using glucose (220 mM) dissolved in a 100 mM acetate buffer pH 5 6 saturated by air at 30 °C according to Rakotozafy et al. (*32*) Activity is expressed in nanokatals (nmol of oxygen consumed per second)

**Hexose Oxidase Assay.** The HOX activity was determined polarographically using glucose (50 mM) dissolved in a 100 mM acetate buffer solution at pH 5 6 saturated with air at 30 °C Activity is expressed in nkat (nmol of oxygen consumed per second in the assay conditions).

**GOX–POD Mixtures.** Evolution of FA in GOX-POD mixtures was analyzed by UV-spectrophotometry (310 nm) according to Garcia et al (*26*) GOX solution used in these experiments was prepared at a concentration of 0 27 mg/mL Wheat POD used in these experiments had a $V_m^{POD}$ value of 5 4 $\mu$kat/mL of enzymatic solution (corresponding to the 0 26 $\mu$kat/mL determined in the standard assay conditions).

The composition of mixtures containing a fixed GOX amount was as follows: 6 7 $\mu$g of GOX (5.1 nkat), 90 $\mu$M FA, 50 mM D-glucose, 10–100 $\mu$L of POD (2.6–26 nkat), and 20 mM $CaCl_2$ in 100 mM acetate buffer pH 5 6 (3 mL final volume)

The composition of mixtures containing a fixed POD amount was as follows: 25 $\mu$L of POD (6.5 nkat), 90 $\mu$M FA, 50 mM D-glucose, 2.7–27 $\mu$g GOX (2.05–20 5 nkat), and 20 mM $CaCl_2$ in 100 mM acetate buffer pH 5 6 (3 mL final volume).

The composition of mixtures for the analysis of the ascorbic acid effect was as follows: 80 $\mu$L of POD (21 nkat), 27 $\mu$g of GOX (20.5 nkat), 90 $\mu$M FA, 20 mM $CaCl_2$, 50 mM D-glucose, and 100 mM acetate buffer pH 5 6 containing from 0 to 500 $\mu$M ascorbic acid (3 mL final volume) Evolution of FA in GOX–POD mixtures containing ascorbic acid was followed both by UV-spectrophotometry and ECD-HPLC, according to Garcia et al. (*26*)

**Comparison of GOX–POD and HOX–POD Mixtures.** Evolution of FA in the mixtures was followed by spectrophotometry according to Garcia et al (*26*) The composition of the mixtures is as follows: 25 $\mu$L of POD (6.5 nkat), 90 $\mu$M FA, D-glucose (from 2 to 50 mM), and 0.42 to 2 6 nkat of GOX or HOX in 100 mM acetate buffer pH 5 6 (3 mL final volume)

## RESULTS AND DISCUSSION

**Glucose Oxidase Characterization.** The effect of glucose concentration on the enzyme activity was determined at three different pHs, at one oxygen concentration (220 $\mu$M). The enzyme affinity toward glucose increases with pH. Conversely, $V_{mappG}^{GOX}$ values decrease when pH increases (Table 1). The best efficiency ($V_{mappG}^{GOX}/K_{mappG}^{GOX}$) of the enzyme was found at pH 5.6

Consequently, experiments were carried out at this pH, hereafter. As glucose concentration in dough is around 4 mM (*35*) and dough pH is between 5 and 6.2 (*32*), it can be adduced that the conditions in dough (regarding the reducing substrate concentration) are not optimal for GOX activity.

Then, the effect of oxygen concentration on the GOX activity was determined. GOX kinetics are Michaelian type with a ping-pong mechanism in agreement with Whitaker (*12*), so the velocity of the reaction is

$$v_G = V_m^{GOX}\frac{[O_2][G]}{K_{mO}^{GOX}[G] + K_{mG}^{GOX}[O_2] + [G][O_2]} \quad (1)$$

where $[O_2]$ = oxygen concentration, $[G]$ = glucose concentration, $V_m^{GOX}$ = maximal velocity, $K_{mO}^{GOX}$ = Michaelian constant toward oxygen, and $K_{mG}^{GOX}$ = Michaelian constant toward glucose.

For a constant oxygen concentration, "apparent" kinetic constants $V_{mappG}^{GOX}$ and $K_{mappG}^{GOX}$ are

$$V_{mappG}^{GOX} = \left(V_m^{GOX}/\left(\frac{K_{mO}^{GOX}}{[O_2]} + 1\right)\right) \quad (2)$$

$$K_{mappG}^{GOX} = \left(K_{mG}^{GOX}/\left(\frac{K_{mO}^{GOX}}{[O_2]} + 1\right)\right) \quad (3)$$

Values previously determined are apparent kinetic constants for an oxygen concentration of 220 $\mu$M (Table 1).

Likewise, for a constant glucose concentration, apparent kinetic constants $V_{mappO}^{GOX}$ and $K_{mappO}^{GOX}$ are

$$V_{mappO}^{GOX} = \left(V_m^{GOX}/\left(\frac{K_{mG}^{GOX}}{[G]} + 1\right)\right) \quad (4)$$

$$K_{mappO}^{GOX} = \left(K_{mO}^{GOX}/\left(\frac{K_{mG}^{GOX}}{[G]} + 1\right)\right) \quad (5)$$

These values were determined by polarography for high glucose concentration. As the stoichiometry of the reaction is one mole of oxygen for one mole of glucose, the maximal consumption of glucose is 220 $\mu$M in an air-saturated solution at 30 °C. For high initial glucose concentrations (e.g., 50 mM in our experiments) this consumption is negligible. Therefore, the equation governing the rate of the reaction is only dependent on oxygen concentration variation

$$v_G = V_{mappO}^{GOX}\frac{[O_2]}{K_{mappO}^{GOX} + [O_2]} \quad (6)$$

To determine the values of apparent kinetic constants, oxygen consumption was analyzed at two enzyme concentrations (78 and 156 $\mu$g/mL) (Figure 1). The initial rates of the reaction were determined and apparent $K_{mappO}^{GOX}$ and $V_{mappO}^{GOX}$ average values (Table 2) were 0.20 ± 0.01 mM and 1.41 ± 0.02 $\mu$kat/mg, respectively.

Using results reported in Tables 1 and 2, the $K_m$ (using eqs 3 and 5) and the $V_m^{GOX}$ (using eqs 2 and 4) values toward glucose and oxygen can be calculated. The $K_{mO}^{GOX}$ value is 0.48 mM (Table 3), which is close to those found by Gibson et al. (*13*) and Vanstroebizen et al. (*36*), namely 0.6 and 0.8 mM. The $K_{mG}^{GOX}$ value is 70 mM and $V_m^{GOX}$ = 3.5 ± 0.1 $\mu$kat/mg (Table 3).

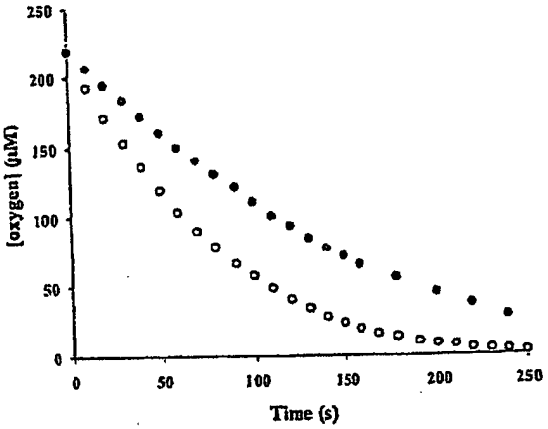


Figure 1. Oxygen consumption by two GOX solutions: 78 $\mu$g/mL (●) and 156 $\mu$g/mL (○), ([glucose] = 50 mM; total volume = 1 mL, 20 $\mu$L of enzyme).

Table 2. $K_{mappO}^{GOX}$ and $V_{mappO}^{GOX}$ for [glucose] = 50 mM and Two GOX Concentrations

| | $K_{mappO}^{GOX}$ (mM) | $V_{mappO}^{GOX}$ |
|---|---|---|
| $E_1$ = 0.156 mg/mL | 0.21 | 1.39 $\mu$kat/mg |
| $E_2$ = 0.078 mg/mL | 0.19 | 1.43 $\mu$kat/mg |

Table 3: Kinetic Constants of GOX toward Glucose and Oxygen

| $K_{mO}^{GOX}$ | $K_{mG}^{GOX}$ | $V_m^{GOX}$ (eq 2) | $V_m^{GOX}$ (eq 4) |
|---|---|---|---|
| 0.48 mM | 70 mM | 3.6 $\mu$kat/mg | 3.4 $\mu$kat/mg |

Table 4: Apparent Kinetic Values of HOX for $[O_2]$ = 220 $\mu$M

| | pH 5.0 | pH 5.6 |
|---|---|---|
| $K_{mappG}^{HOX}$ (mM) | 0.6 | 1 |
| $V_{mappG}^{HOX}$ (nkat/mL) | 24 | 34 |
| $V_{mappG}^{HOX}/K_{mappG}^{HOX}$ | 40 | 34 |

As $CaCl_2$ is an activator of wheat germ POD (*26*), it was added in the GOX–POD mixtures. The addition of $CaCl_2$ from 0 to 30 mM at pH 5.6 did not have any effect on the consumption of oxygen by GOX (results not shown).

**Hexose Oxidase Characterization.** HOX exhibited a maximum of activity at pH 5.6, and few differences were detected between pH 5.0 and 5.6 (results not shown). The effect of glucose concentration was analyzed at pH 5.0 and 5.6 at one oxygen concentration (220 $\mu$M). Affinity toward glucose decreased with increasing pH, but conversely $V_m^{HOX}$ values increased with pH (Table 4). The best efficiency was found at pH 5.0, and a loss of only 16% of this value was determined when pH was increased up to 5.6. According to these results, it can be assessed that conditions in dough are not far from optimum for HOX activity (regarding to the glucose concentration). Moreover, HOX can use other sugars as substrates in dough (*5*).

The addition of calcium chloride (from 0 to 30 mM) at pH 5.6 did not have any effect on the consumption of oxygen by HOX (not shown).

**Glucose Oxidase–Peroxidase Association.** Assuming that the endogenous glucose concentration in dough is approximately

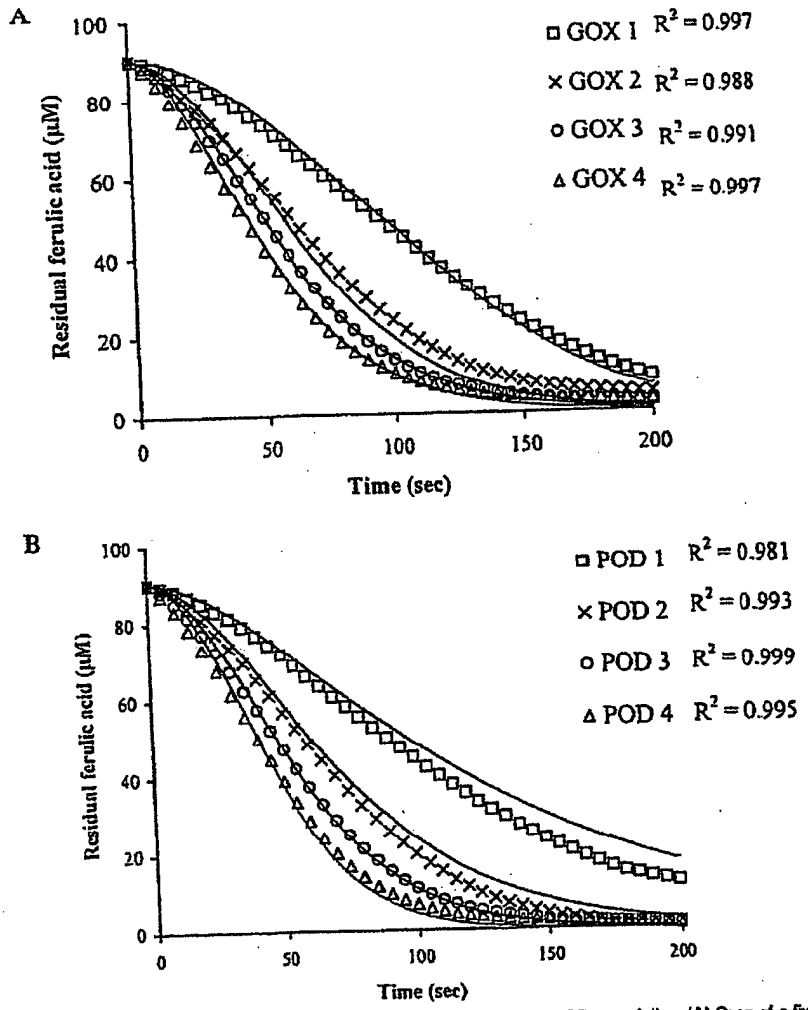


Figure 2. Comparison between experimental (points) and theoretical (lines) data for a GOX–POD association. (A) Case of a fixed POD amount (25 μL) and variable GOX amounts. GOX 1 = 2.7 μg; GOX 2 = 5.4 μg; GOX 3 = 8.1 μg; GOX 4 = 10.8 μg. (B) Case of a fixed GOX amount (6.7 μg) and variable POD amounts. POD 1 = 10 μL; POD 2 = 20 μL; POD 3 = 30 μL; POD 4 = 40 μL.

4 mM (*35*), the oxidation of FA in GOX–POD systems was analyzed at 2 and 5 mM of glucose. $H_2O_2$ production by GOX was monitored by ECD–HPLC according to Garcia et al. (*26*). At either glucose concentration, sufficient $H_2O_2$ was produced to activate POD and oxidize all the FA present in 600 (2 mM glucose) or 400 (5 mM glucose) seconds (data not shown). However, for the purpose of the modeling, glucose concentration was set at 50 mM to have a negligible variation of the reducing substrate concentration during the course of the reaction. FA consumption was analyzed for different GOX/POD ratios. FA consumption was followed by UV-spectrophotometry and confirmed by ECD-HPLC, according to Garcia et al. (*26*).

Incubations were made with FA (90 μM), a fixed POD amount (6.5 nkat) and increasing GOX amounts (Figure 2A). Then, the reciprocal experiment was made (Figure 2B). In all the experiments, FA consumption rates increased with increased enzyme concentrations and curves were all sigmoidal. This is due to the fact that, at the beginning of the reaction POD activity is equal to zero by a lack of hydrogen peroxide and during the course of the reaction, the rate increases as GOX produces $H_2O_2$. At the end of the reaction, the rate decreases, because the FA concentration becomes a limiting factor. Maximal rates were measured for each curve and values were plotted against GOX amount (Figure 3A) or POD amount (Figure 3B). In both cases, a plateau was reached. For a constant POD concentration and increasing amounts of GOX, the plateau value was lower than the control (same amount of POD in the presence of FA and 500 μM $H_2O_2$). The oxygen content of the medium obviously limited the production of $H_2O_2$ by GOX to 220 μM. For the same reason, when GOX concentration was constant, the rates were always slower than the rates in the control.

**Mathematical Modeling of GOX–POD Association.** In a first step, the equation describing the evolutions of oxygen and hydrogen peroxide relative to GOX activity was established. Oxygen consumption by GOX in a medium containing 50 mM glucose follows a Michaelian kinetic with one substrate, in

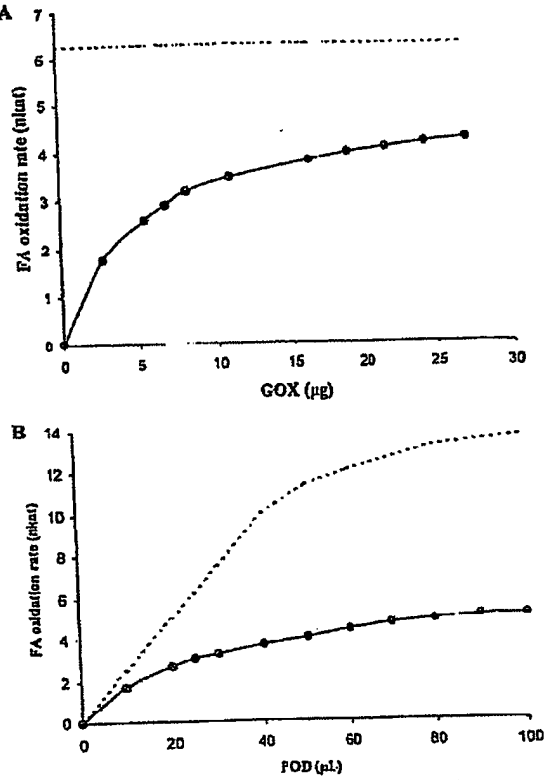


Figure 3. Evolution of FA oxidation rates in a GOX–POD mixture: ···, control (500 μM $H_2O_2$ + POD), —●— GOX + POD (A) For increasing GOX amounts (POD = 25 μL). (B) For increasing POD amounts (GOX = 6.7 μg).

which oxygen is limitant (see above). The equation is

$$v_t^{GOX} = -\frac{[O_2]_t - [O_2]_{t-1}}{dt} = V_{mappO}^{GOX}\frac{[O_2]_{t-1}}{[O_2]_{t-1} + K_{mappO}^{GOX}} \quad (7)$$

where $v_t^{GOX}$ is the GOX oxygen consumption rate at time $t$.

The oxygen content in the medium at time $t$ ($[O_2]_t$) can be calculated from the oxygen content at time $t-1$ ($[O_2]_{t-1}$), using the equation

$$[O_2]_t = [O_2]_{t-1} - V_{mappO}^{GOX}\frac{[O_2]_{t-1}}{[O_2]_{t-1} + K_{mappO}^{GOX}}dt = [O_2]_{t-1} - v_t^{GOX}dt \quad (8)$$

As the stoichiometry of the reaction is one mole of oxygen consumed for one mole of hydrogen peroxide formed, the hydrogen peroxide production is equivalent to oxygen consumption, so the $H_2O_2$ content in the medium at time $t$ is

$$[H_2O_2]_t = [H_2O_2]_{t-1} + V_{mappO}^{GOX}\frac{[O_2]_{t-1}}{[O_2]_t + K_{mappO}^{GOX}}dt = [H_2O_2]_{t-1} + v_t^{GOX}dt \quad (9)$$

In a second step, the equation describing the evolution of substrates (FA and $H_2O_2$) relative to POD activity was established. POD kinetics are Michaelian type with a ping-pong mechanism, and kinetic constants were previously determined (*26*)

$$v_t^{POD} = \frac{[FA]_t - [FA]_{t-1}}{dt} = V_m^{POD}\frac{[H_2O_2]_{t-1}[FA]_{t-1}}{K_{mFA}[H_2O_2]_{t-1} + K_{mH_2O_2}[FA]_{t-1} + [H_2O_2]_{t-1}[FA]_{t-1}} \quad (10)$$

where $v_t^{POD}$ is the FA consumption rate by POD at time $t$, and with $K_{mFA} = 1.5$ mM, $K_{mH_2O_2} = 1.2$ mM, and $V_m^{POD} = 5.4$ μkat/mL.

Ferulic acid content at time $t$ ($[FA]_t$) can be determined using contents at time $t-1$ ($[FA]_{t-1}$ and $[H_2O_2]_{t-1}$) and according to the relationship

$$[FA]_t = [FA]_{t-1} - V_m^{POD}dt\frac{[H_2O_2]_{t-1}[FA]_{t-1}}{K_{mFA}[H_2O_2]_{t-1} + K_{mH_2O_2}[FA]_{t-1} + [H_2O_2]_{t-1}[FA]_{t-1}} = [FA]_{t-1} - v_t^{POD}dt \quad (11)$$

The stoichiometry is two moles FA for one mole $H_2O_2$ and it is assumed that there is no inhibition by any of the substrates. Likewise, peroxidase-catalyzed consumption of $H_2O_2$ can be calculated using content at time $t-1$ ($[H_2O_2]_{t-1}$) and the equation

$$[H_2O_2]_t = [H_2O_2]_{t-1} - \frac{1}{2}V_m^{POD}dt \times \frac{[H_2O_2]_{t-1}[FA]_{t-1}}{K_{mFA}[H_2O_2]_{t-1} + K_{mH_2O_2}[FA]_{t-1} + [H_2O_2]_{t-1}[FA]_{t-1}} = [H_2O_2]_{t-1} - \frac{1}{2}v_t^{POD}dt \quad (12)$$

When both GOX and POD are in the medium, the $H_2O_2$ concentration at time $t$ can be calculated from the value of $H_2O_2$ content at time $t-1$ ($[H_2O_2]_{t-1}$) and the sum of $H_2O_2$ produced by GOX ($v_t^{GOX}$) and $H_2O_2$ consumed by POD ($^1/_2\ v_t^{POD}$)

$$[H_2O_2]_t = [H_2O_2]_{t-1} + v_t^{GOX}dt - \frac{1}{2}v_t^{POD}dt \quad (13)$$

The different equations (11–13) allow the calculation of oxygen, $H_2O_2$, and FA concentrations during the course of the reaction.

**Comparison Between Model and Experimental Data.** Equations 11–13 were used for the modeling of FA oxidation under different experimental conditions.

Two sets of experiments were carried out. First, a fixed GOX amount (6.7 μg) was associated with increasing POD amounts (10–100 μL). Then, a fixed POD amount (25 μL) was associated with increasing GOX amounts (2.7–27 μg).

For each experiment, $V_m$ values for GOX and POD needed to be calculated.

In the case of GOX, values needed for the calculation of $V_{mapp}^{GOX}$ (in μkat for *1 liter of reaction solution*) were $K_{mappO}^{GOX}$, $V_{mappO}^{GOX}$ (in μkat for *1 mg of enzyme powder*) (both in Table 2), the enzyme amount (in μg) and the glucose concentration (50 mM)

Table 5. POD and GOX Amounts and Corresponding $V_m$ Values Used for Modeling the GOX–POD Association

| POD amount | | GOX amount | | $V_m^{POD}$ | $V_{mapp}^{GOX}$ |
|---|---|---|---|---|---|
| μL | (nkat) | μg | (nkat) | (μkat/L of rxn soln)[a] | (μkat/L of rxn soln)[b] |
| 25 | 6 5 | 2 7 | 2 05 | 45 | 1 3 |
| | | 5 4 | 4 1 | 45 | 2 6 |
| | | 7 1 | 6 15 | 45 | 3 9 |
| | | 10 8 | 8 2 | 45 | 5 2 |
| 10 | 2 6 | 6 7 | 5 1 | 18 | 3 25 |
| 20 | 5 2 | | | 36 | 3 25 |
| 30 | 7 8 | | | 54 | 3 25 |
| 40 | 10 4 | | | 72 | 3 25 |

[a] Calculated using $V_m^{POD}$ = 5 4 μkat/mL of enzyme [b] Calculated for $V_m^{GOX}$ = 3 5 μkat/mg (Table 3) and using eq 4

In the case of POD, values needed for the calculation of $V_m^{POD}$ (in μkat for *1 liter of reaction solution*) were the volume of enzyme used (in μL) and $V_m^{POD}$ (in μkat for *1 mL of enzyme solution*). Kinetic constants previously determined for POD (*26*) were used: $K_{mFA}$ = 1 5 mM, $K_{mH_2O_2}$ = 1 2 mM.

Calculated values of $V_m^{POD}$ and $V_{mapp}^{GOX}$ used for the modeling are recorded Table 5.

The modeling of FA oxidation for each GOX–POD association was compared with the experimental data (Figure 2, parts A and B). A good correlation was found for all the associations ($R^2$ average values are 0.992), but some gaps appear at the end of the reaction. An inhibition due to an excess of one of the substrates when the other one is at low concentration (as it often happens for peroxidase) may be responsible for this anomaly, and this has not been integrated in the mathematical model. Another possibility may be that stoichiometry variation occurs, due to the oxidation of other substrates (e.g., FA dimers) by POD during the course of the reaction (*37*). Finally, as our system is not totally hermetic, external oxygen supplies ingress, especially for high GOX amounts or for periods after 60 s. Under these conditions calculated curves under-estimate FA consumption because $H_2O_2$ production by GOX was under-estimated. In effect, with external supplies, oxygen concentration available could be more important than the concentration used for the model.

Nevertheless, the modeling of FA evolution appears possible (using equations 11,−13) in a GOX–POD system, at least in the experimental field studied.

**Ascorbic Acid Effect on GOX–POD Association.** The effect of ascorbic acid (AA) was determined by UV-spectrophotometry and ECD-HPLC. This compound delays the oxidation of FA and HPLC data confirm that during the lag period, AA and $H_2O_2$ are the only species consumed (not shown). When all AA is consumed, FA consumption begins. This result can be explained by a coupled oxidation mechanism, as previously described in the case of FA oxidation by the system wheat POD/$H_2O_2$ (*26*). AA is rapidly oxidized into dehydroascorbic acid by the phenoxy radicals produced by POD in the presence of $H_2O_2$ leading to an immediate regeneration of FA.

This result has a consequence in baking technology. It is generally accepted that the improving effect of GOX is due to the $H_2O_2$ produced, which can then be used by POD to gel pentosans. It seems that the use of formulations containing both AA and GOX is not judicious, as AA will delay the effect of POD

**Hexose-Oxidase–Peroxidase Association.** *Comparison with GOX–POD Association* To compare the activation of POD by the $H_2O_2$ produced by GOX or HOX, the amounts of each enzyme necessary to obtain the three initial oxygen consumption velocities (2.6, 1 5, and 0 42 nkat) were determined for three glucose concentrations, namely 2, 5, and 50 mM (Table 6).

Table 6. HOX and GOX Amounts Necessary to Obtain the Same Initial Oxygen Consumption Velocities

| [glucose] used for the assay (mM) | GOX amount (μg) | HOX volume (μL) | equivalent activity (nkat) |
|---|---|---|---|
| 50 | 3 6 | 78 | 2 6 |
| 5 | 7 1 | 55 | 1 5 |
| 2 | 5 | 20 | 0 42 |

Table 7. Time Necessary to Consume 45 μM of FA ($t_{1/2}$) for Each GOX–POD or HOX–POD Association at Different Glucose Concentrations

| | $[glucose]_{mM}$ (mM) | GOX amount (μg) | HOX volume (μL) | $t_{1/2}$ for GOX–POD (sec) | $t_{1/2}$ for HOX–POD (sec) |
|---|---|---|---|---|---|
| 1 | 50 | 3 6 | 78 | 74 | 55 |
| 2 | 5 | 3 6 | 78 | 164 | 60 |
| 3 | 2 | 3 6 | 78 | 286 | 67 |
| 4 | 5 | 7 1 | 55 | 105 | 71 |
| 5 | 2 | 5 | 20 | 246 | 157 |

A decrease in glucose concentration and the initial oxygen uptake velocity has different effects on HOX and GOX: GOX amounts need to be increased, whereas HOX volumes need to decreased. This can be easily explained by the kinetic constants of each enzyme; HOX has much more affinity toward glucose than GOX has (Table 1 and Table 4).

A set of experiments were then carried out to compare the GOX–POD and the HOX–POD associations. Several amounts of GOX or HOX were associated with a fixed amount of POD (6 5 nkat), and the oxidation of ferulic acid was followed by the absorbance decrease at 310 nm. Table 7 shows the time ($t_{1/2}$) necessary to consume half of the initial amount of FA present in the mixtures (45 μM, equivalent to a decrease of 0.4 absorbance units at 310 nm) for different glucose concentrations

The $t_{1/2}$ were always shorter for HOX than for GOX, indicating that HOX was the most efficient one. A decrease of glucose concentration from 50 to 2 mM had much more effect on GOX than on HOX activity (comparison of lines 1 and 3, Table 7): $t_{1/2}$ were lengthened 4 times for GOX–POD association versus 1.2 times for HOX–POD association. This could easily be explained by the fact that the $H_2O_2$ production rate was decreased 9 times for GOX, whereas it was only 1 5 times lower for HOX. Again, this effect can be attributed to the differences in affinity toward glucose for the two enzymes

Finally, experiments were conducted with the same glucose concentration (2 or 5 mM) but with adapted amounts of GOX and HOX to obtain in both cases the same $H_2O_2$ production rate (lines 4 and 5, Table 7). HOX was still more efficient compared to GOX as $t_{1/2}$ values were always lower in the case of HOX–POD association.

A comparison of the oxygen uptake by GOX and HOX showed that if the initial rate is equivalent for both enzymes, the oxygen consumption slowed more rapidly with GOX than with HOX (Figure 4) This phenomenon is not due to an inhibition by hydrogen peroxide since both enzyme kinetics are not affected by $H_2O_2$ in the concentration range of this study (not shown); therefore, it can be assumed that the affinity for oxygen is higher for HOX than GOX.

**Conclusions.** Wheat peroxidase in association with GOX or HOX is able to oxidize FA The hydrogen peroxide produced

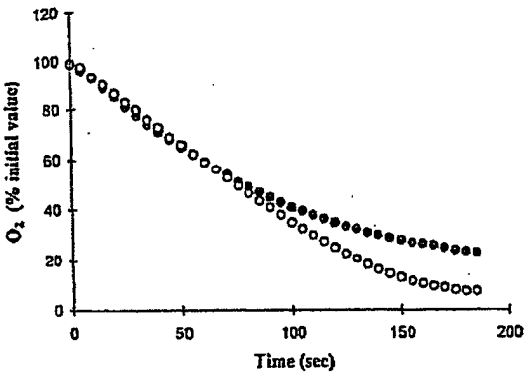


Figure 4. Comparison of the oxygen consumption by 2.6 nkat of GOX (●) or HOX (○), ([glucose] = 50 mM).

by GOX or HOX can activate POD (even for low glucose concentrations). Accordingly, part of the effect of GOX in bread making can be attributed to endogenous POD activation as suggested by Vemulapalli et al. (*16*) and Ameille et al. (*18*). Likewise, part of the effect of HOX in bread making could be attributed to POD-catalyzed pentosans gelation as proposed by Poulsen and Bak Hostrup (*5*).

A mathematical model for the GOX−POD association in oxidizing FA is proposed, which describes the evolution of the different reactants ($H_2O_2$, FA, and oxygen) during the course of the reaction. A good correlation with experimental data validated the model for the experimental field studied.

## ABBREVIATIONS USED

AA, ascorbic acid; ECD, electrochemical detector; FA, ferulic acid; GOX, glucose oxidase; HOX, hexose oxidase; HRP, horseradish peroxidase; POD, peroxidase; SH, sulfydryl group; S−S, disulfide bridges

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INDUSTRIAL Enzymology SECOND EDITION

# Chapter 2.5

## *Baking*

T. Godfrey

## 2.5.1 INTRODUCTION

Baking is one of the three oldest biotechnology industries and has evolved with the development of civilized society to a very sophisticated and diverse activity. As a staple in the diet of almost all communities the production of bread is justifiably subject to thorough regulation in most countries. These regulations are different from country to country but in all cases are in place to ensure good quality and wholesome food at prices that should be within the reach of virtually all citizens.

The introduction of industrial enzymes from the early part of this century allowed the baking industry to extend its acceptance of raw cereals to include wheat that had been subject to adverse growing conditions, varieties of wheat that would grow in the consumer locality, to other species such as maize, rice, rye, oats, and barley. Some breads are also produced in very small regions from yet other starch sources. For yeast-raised bread production these non-wheat cereals are frequently supplemented with some wheat flour, with the possible exception of rye.

This chapter will concentrate on the aspects of baking production that are influenced by added enzymes. For detailed consideration of the characteristics of cereals and flours made from them, together with more authoritative discussion of the production of baked goods the reader is encouraged to consult items mentioned in Further Reading listed at the end of the chapter. For the purposes of this presentation the main discussion will focus on the production of bread from wheat.

## 2.5.2 THE MAIN DOUGH PRODUCTION PROCESSES

While there are many speciality processes involved in craft baking, there are four main processes in use for industrial scale production of bread. Each differs in the way the dough is prepared prior to baking. The baking step is influential on the final bread character, being influenced by the temperatures, humidity, and time employed. It is in the dough stages that many of the factors affecting the use and selection of enzymes are encountered.

Considerable effort is exerted by industrial bakeries to achieve as rapid and efficient a process as possible while they try to deliver consistent and desirable products.

### 2.5.2.1 Sponge and dough

Part of the flour is mixed with the yeast and any additives and enzymes and blended with water to produce a dough. This is allowed to ferment for an extended period of up to 4 h. The remainder of the flour and any salt required is now added and the mix allowed a short mixing and fermentation followed by cutting and shaping. The pieces are proofed. The total time for this process will be 6–7 h.

### 2.5.2.2 Straight dough

All the ingredients are mixed together to form a dough and this is fermented for up to 3 h. Proofing, cutting and shaping and re-proofing takes the total time up to 5–6 h. This can be shortened by about 1 h if the mixing time is extended and moving directly to proofing without a fermentation hold.

### 2.5.2.3 Continuous dough production

Flour is continuously added to a prefermented liquid consisting of all the water with the yeast, salt, and additives, and mixed to form a dough. Proofing, cutting, and sometimes re-proofing will give a total time of 4–5 h.

### 2.5.2.4 Chorleywood process

This process is operated either as a batch or continuous and replaces the fermentation time by a vigorous mixing of the dough in the presence of oxidants (e.g. potassium bromate).

Cutting, shaping and proofing make the total time between 1 and 2.5 h. The activated dough process is a version of the Chorleywood process with the addition of L-cysteine hydrochloride to accelerate the breaking of disulphide bonds in the protein matrix. This allows a further reduction in process time.

## 2.5.3 THE ACTION OF ADDITIVES AND PROCESSING AIDS

Fungal amylases and sometimes bacterial amylases, proteinases and enzyme active soya flour are well established contributors to the baking performance. Recent advances in our understanding of the dough-forming and overall baking processes at the molecular level have focused attention on further improvements that can be achieved by the application of more specifically tailored enzymes to modify components of the flour that are neither starch nor protein.

A variety of chemicals are also used to establish speed, economy and quality in the industrial process. These will not be discussed in detail in this chapter, but referred to as their relevance to an enzyme concept occurs.

A worldwide pressure from the consumers has pressed the baking industry to remove the chemical load from its products. While most of this pressure is based on a general desire to have less chemicals in our diets, regulatory views based on investigations have begun to impact on some of them.

In Europe it is now general that potassium bromate is prohibited in bread. This ban is extending to other countries, and while not actually banned in the USA there is a request from the Food and Drug Administration (FDA) to reduce its use as much as possible. Many US bakers have in fact removed it from their formulas. In the biscuit industry there has been a long-standing use of sulphur dioxide to reduce the elasticity of gluten. This is now generally banned and may be substituted by the suitable use of proteinases (Section 2.5.6). The use of other chemical compounds continues but under close scrutiny: azodicarbonamide, ascorbic acid, chemically produced mono- and diglycerides, and the Data esters (e.g. stearoyl lactylate, diacetyl tartaric esters of monoglycerides), benzoyl peroxide, chlorine dioxide, ammonium and potassium persulphate, monocalcium phosphate, calcium or sodium propionate. The permitted levels of these chemicals all fall in the parts per million (ppm) range. Soya flour is also added to many recipes. The amount is not subject to regulation, but it is typically no more than a few percentage points, as substantial additions would dramatically alter the bread character. The reasons for adding these various compounds relate to the theories describing dough development and baking.

The general requirement is that a dough should be acceptable for handling, including fast mechanical systems, and be able to initiate and sustain a gas production that will raise the volume while retaining a good structure, and not collapse or otherwise degrade when baked, or at some time after baking. There are also the requirements of taste and texture, crumb and crust colour, and the overall keeping properties to consider. It is well recognized that the starch component of the flour is the source of the sugars for the fermentation, and this may be supplemented with small amounts of added sugars. The creation of carbon dioxide is only important in the proof stage and its retention is critical to the bread quality. It is also considered that yeast activity contributes to flavour, although crust formation is probably more significant.

Starch modification will be discussed later (see also Section 2.5.4)

### 2.5.3.1 Protein oxidation

Most of the opinions about dough development are directed at the behaviour of the very particular proteins of wheat. Typically, the wheat flour will have 10.5–13 per cent protein content. This protein is comprised of large and small molecular sized types. The small proteins (albumins and globulins) are water soluble. The large proteins (gliadins and glutenins) are insoluble. Exactly what happens to the large proteins when water is added to the flour is very much a matter for discovery and there are at least as many opinions on the action of the various additives as there are centres of excellence studying the subject. What is certain is that the spatial structure of these proteins as they are hydrated by water is subject to change. There are several bond types that establish this arrangement and the strongest is the disulphide bond between sulphydryl groups of different cysteine molecules in different regions of the same protein strand or in different strands.

Under hydration and mixing forces, distortion and flexing of the proteins may occur. The creation of new disulphide bondings and the breaking of others alters the overall structural relationships. It is most often stated that the result in a dough is the reduction in the number of bonds between regions of a single protein strand, and an increase in bonding between different strands. This new network of protein will be the basis of the gas-tight seal that forms the small micelles that expand as carbon dioxide is generated by the yeast. These 'protein membranes' must be able to expand without bursting, and to set firm in the oven to form the texture and structure of the bread. It is believed that the most important new bonds for gas retention are those between the large insoluble proteins. The traditional and slow process of doughs generally created a suitable internal structure; however, modern high-speed mixing operations do not produce satisfactory results. The introduction of oxidizing substances is based on the expectation that the soluble small proteins will be subject to rapid oxidation to form interactive bonds and so eliminate them from the apparent competition for new bond formation. There will thus be a promotion of interactive bonding between the larger protein strands. There are four chemicals that have been used to provide an encouraging environment to promote this shift of bonding: ascorbic acid, azodicarbonamide, potassium bromate, and L-cysteine.

### 2.5.3.2 Emulsifiers

The usual function attributed to these substances is that they combine with the flour proteins and strengthen them so that they can expand and retain the generated gas. This is particularly claimed for the Datem esters. The use of monoglycerides is largely for the increased

shelf-life they impart. This is possibly by some interaction between fats and starch.

### 2.5.3.3 Soya flour

Provided it has not been subjected to heat, soya flour contributes a bleaching effect and also an oxidative effect. Both these effects are produced by the enzyme lipoxygenase. It uses atmospheric oxygen to oxidize unsaturated fats (e.g. linoleic acid) to form lipid peroxides. These oxidize the coloured components of the flour (e.g. carotenoids) turning them to colourless compounds. The improvement in dough character is attributed to an oxidative attack on the small soluble proteins of the flour in similar fashion to the chemical oxidants. In addition it is proposed that the lipid peroxides are also able to reach areas of gluten that are hydrophobic and so act where the chemical oxidants cannot. It is also noted that the soya lipoxygenase improves gluten strength at higher work input levels from the mixer than do the chemical oxidants. This broadens the tolerance range for mixing compared with chemical oxidants and so reduces the sensitivity to work in the processing. Considerable work is currently in hand to develop sources of lipoxygenase that can be used in this way without the requirement to dilute the wheat flour with large quantities of soya flour. Having established the probable function of the chemicals used in bread making the various enzymes available and their contributions to the overall objectives will be discussed.

## 2.5.4 AMYLASES

In order that yeast may generate carbon dioxide to raise the dough it requires a supply of fermentable sugar. There is a little fermentable sugar, mostly maltose, in bread flour. It rarely exceeds 0.5 per cent and this is insufficient for bread making. Wheat flour contains both alpha and beta-amylases which can activate the dough to produce more fermentable sugars. These may be unbalanced and also overactive in some cases. In seasons of high humidity prior to grain harvest the wheat has large amounts of alpha-amylase and its use leads to poor and sticky dough character and also sticky crumb with high colour in the bread. Ungerminated, the wheat has large amounts of beta-amylase and low levels of alpha-amylase. The result is inadequate amounts of fermentables. It is therefore quite usual to supplement the flour by adding fungal alpha-amylase at the mill.

The amylolytic action is greatest on damaged starch granules and these may be produced in excess if the milling is not well regulated. Hard wheats will tend to risk damage more than soft wheats. Early in the baking stage whole starch granules are modified and become easily attacked by alpha-amylase.

The amylases also act in the dough stage, particularly on damaged starch granules.

### 2.5.4.1 Amylase action

Cereal beta-amylase yields maltose from damaged starch granules. This is slowly fermented and can only be fermented after the yeast has been induced by glucose molecules. Flours with low natural alpha-amylase activity to generate glucose will have a poor fermentation character. This can be corrected by the addition of another amylase, glucoamylase, or direct addition of glucose. Both of these have the potential to adversely affect the dough and the finished product. It is usual to restrict their use to speciality products such as rusk. Rye flours are more susceptible to amylase attack than wheat flours and their action is often stimulated by preparing the dough with hot water.

Alpha-amylase action produces glucose and dextrins. The dough character is altered by the hydrolysis of damaged starch granules which are rapidly and readily hydrated. It is generally thought that the dextrins produced by amylase action play a part in the overall hydration of the dough, and to a small extent increase the shelf-life of the final product. They may also contribute to texture and mouthfeel in the final product. The overall pattern of amylase activity during the baking stage depends on the proportions of the different types of amylase involved and their respective deactivation temperatures. The fungal amylases are the predominant choice for baking supplementation, but bacterial amylases are sometimes considered. The bacterial type will be dealt with first.

### 2.5.4.2 Bacterial amylases

Initially tried as replacements for expensive and rather variable malt extract sources of additional amylase, the bacterial amylases were found to have too great a heat tolerance for most conventional bread making. As they were not inactivated until very late in the baking stage they tended to be overactive, and this gives rise to considerable problems in ensuring very precise dose controls. These enzymes, from *Bacillus subtilis* and more recently from *Bacillus licheniformis*, were developed as powerful starch hydrolysing enzymes for the starch/syrup and textiles industries. The usual commercial products are liquids which are not suited to use in baking, and the powder versions are generally of such high activity that a controlled predilution is essential to control the dose into a dough formulation. Even small levels of overdosage can create sticky doughs and a poor final texture. The bacterial amylases are far lower in price than fungal amylases, and they are very fast acting. These are potential benefits for the baker, but the control problem outweighs the benefit. The most com-

mon current use in baking is to create a soft, sweet and sticky final product such as honey cake and 'malt loaf'.

In the search for increased shelf-life without recourse to high levels of chemical additives there have been recent promotions of new and carefully selected bacterial amylases. By selecting away from the starch liquefying strains, and concentrating on the maltogenic properties, these new types are thought to function by either reducing the tendency of starch to crystallize (retrogradation) on cooling, or to actually hydrolyse the retrograded starch granules preferentially. The actual result is a longer lasting good taste and texture and an extended period of retained loaf spring. These characteristics impart a general acceptance of quality after longer storage times.

The Catamyl range (Biocatalysts) are selected blends of conventionally produced amylases, while Novamyl (Novo Nordisk) is from a genetically modified strain of *Bacillus subtilis*. In practice, these products demonstrate that they do not suffer from problems of precise dosing, and overdosing is virtually impossible. Typical economic use rates fall between 60 and 120 ppm based on the total flour. The length of the extension to shelf-life is not easily predicted as it is affected by many factors in the overall dough formulation, the processing method and the baking conditions. It may be assumed that at least 2 days extra shelf-life is required for a cost effective use of these enzymes.

### 2.5.4.3 Thermal inactivation of amylases

The inactivation temperatures of amylases may be given by suppliers and are based on laboratory tests. These may not be the actual inactivation temperatures within a baking operation. Although not absolutely accurate, the following values for the inactivation temperatures in typical baking situations may be used to interpret the effects of cereal and added amylases. Starch gelatinization is going to start at approximately 58 °C (Table 2.5.1).

The thermal inactivation is not linear and approximately half the activity is gone by the time the temperature reaches about one-third of the ranges given.

Table 2.5.1 The regions of thermal inactivation of amylases under baking conditions

| Amylase | Temperature °C |
|---|---|
| Cereal beta-amylase | 55–75 |
| Fungal alpha-amylase | 55–80 |
| Cereal alpha-amylase | 70–85 |
| Bacterial alpha-amylase | 75–90+ |

### 2.5.4.4 Fungal alpha-amylase

It is conventionally accepted that small quantities of fungal alpha-amylase are added to baking flours and this is usually carried out at the flour mill. The most common unit of activity for commercial fungal alpha-amylases is the SKB unit, and typically baking flours are supplemented with between 5 and 15 SKB units of amylase per 100 kg flour. A more historic unit, the Farrand unit, is still used. The relationship between the two units cannot be declared with precision as the assays are not quite identical. As there are so very many different supply sources of this type of enzyme, there can also be differences in the relative activities by the two methods because of small variations in the way the different amylases break down the starch. It is often necessary to adopt a value for relating these two units because most fungal amylases will be sold on the SKB unit basis. Anything between 113 and 116 Farrand units to one SKB unit are found in use. There are other important criteria on which to base an alpha-amylase selection. These include an understanding of the contribution of enzyme side activities to the baking process.

Many fungal alpha-amylase preparations are now offered at very high SKB activities. Many will be greater than 100 000 SKB per gram, whereas only a few years ago it was unlikely to be offered greater than 50 000 SKB per gram of enzyme preparation. It is important to recognize that to obtain very high activity the enzyme preparation will have been subjected to concentration purification or the fermentation and the strain of fungus may have been manipulated to elevate the activity yield.

When side activities prove to be important to the baker, these very high amylase activities can result in reduced overall levels of side activities or changed relative proportions between them.

Typical examples of fungal alpha-amylases for baking will be found in the Amylase 11 P range (Biocatalysts). In cases where wheat has been subject to adverse conditions prior to harvest there may be excess alpha-amylase. This is usually adjusted by blending the flour with other flours with much lower amylase content.

Table 2.5.2 Key objectives of the industrial baking process

| | |
|---|---|
| Final and stable volume | Crust colour and texture |
| Dough consistency | Texture and taste |
| Mechanical handling | Shelf-life extension |
| Reduced energy input | Reduction of process time |
| Production economy | Raw materials flexibility |
| Conformity to regulations | Customer satisfaction |

## 2.5.5 ENZYMES OTHER THAN AMYLASES

To assist in the understanding of the roles for other enzymes in the dough-forming and baking process the overall objectives of the baker can be restated (Table 2.5.2). Whatever modifications to the ingredient formulation and the processing methods may be made there will be changes in the behaviour of the dough and the baked out product character. To be acceptable they will have to be within the regulations, use economic levels of process aids and additives, and correspond to prevailing economic pressures in line with competitive and customer forces. The complex interaction of changes designed to influence one particular characteristic will usually only be seen in the results and may not readily be predicted. The introduction of other enzymes than amylases will usually be to make a specific biochemical modification of some part of the ingredients but will also usually be found to influence other characteristics as well. These changes can be positive and accumulative. They can also be unacceptable. A full recipe baking test is essential when introducing any new component or change in processing.

Throughout this chapter, apart from the direct application of fungal amylases to flour, all the mentioned commercial enzymes and enzyme preparations will be used in amounts that must be determined by baking trials, but the dosage will typically fall in the range 30 to 250 ppm based on weight of flour.

### 2.5.5.1 Enzymes acting on carbohydrates other than starch

Apart from a small contribution to the hydration of the dough and possibly being the source of some of the characteristic texture, and staleness, starch is primarily the bulk material of bread. It exerts very little functionality compared with the other components. It has been stated that there are cereal amylases in wheat flours. There are also a large number of other carbohydrases, but these are low in activity and most of them are more heat sensitive than the amylases. These are therefore considered not of great significance in baking. It has been noted that the fungal amylases presented for baking applications, while usually being characterized by a stated level of amylase activity, bring with them a variety of other enzymes in variable amounts. These will be:

- Carbohydrases (cellulase, hemicellulase, pentosanase, glucanase)
- Proteinases and peptidases and lipases.

The selection of a particular supply of fungal amylase may provide an especially suitable selection and potency of these additional enzymes and their presence may explain why a supply, apparently consistent in its alpha-amylase value, will not perform consistently when used. Only a very small number of the suppliers of fungal amylase will also be offering declared, quantified and standardized levels of these other enzymes. Those that do so are likely to provide preparations of particular value to the baker.

Hemicellulose is a term used to describe the non-starch carbohydrate polymers from a botanical material that are insoluble in water. Pentosans are considered to be the similar residues that are water soluble. This distinction appears not to be used when these enzymes are considered for baking or by the regulating authorities. It appears that the common agreement is to use hemicellulose to describe all these residuals. Polymers containing only glucose, but with some other compounds in association, are termed glucans, and there are small amounts of these in wheat flour.

### 2.5.5.2 Cellulases

There is little cellulose remaining in a well-prepared baking flour. In combination with the pentosans and hemicellulose the total will be less than 6 per cent of the flour weight.

The action of the cellulases that are present as side activities of amylases will be to reduce the average size of cellulose polymers if the cellulose is present as a pure polymer. Typically there are fragments of insoluble fibrous material that consist of cross-linked and heterogeneous polymers of both glucose and a number of pentose sugars. These are subject to cellulase attack only so far as a section of cellulose (glucose polymer) is exposed.

### 2.5.5.3 Glucanases

There are other smaller polymers of glucose, the glucans, which are rapidly hydrated, and tend to form gels. These gels can contribute to dough stickiness and poor machinability. The presence of some glucanase activity in the dough contributes to effective control of this condition.

### 2.5.5.4 Pentosanases (hemicellulases)

The polymers of the pentose sugars (mainly arabinogalactan and arabinoxylan) contribute up to 4 per cent of the flour weight. The soluble fraction will be between 1 and 2 per cent of the flour weight and may contribute to the creation of gums and stickiness problems. If the gums are hydrated and combined with the protein matrix of the dough there is some indication that this will increase the strength and stretching ability of the gas cell structures. This will improve the retention of the gas and reduce the potential for gas cell breakdown and merging to produce unwanted larger

holes in the final bread. If the balance between this gas retention benefit and the sticky dough penalty can be created and maintained then the action of these enzymes can be of value. The insoluble hemicellulose fraction is inert except for a slow hydration. There are indications, however, that different types of hemicellulase (pentosanase) enzymes can bring into play some highly valuable attributes by altering the structure. There are long polymers of pentose sugars which are cross-connected by bonds between them, and have some side branches. Hemicellulases are found that will specifically break the bridging and branching bonds so releasing the individual linear polymers. These will then be able to move relative to each other and contribute to the expansion strength of gas cells and have the potential to reduce the use rate or replace chemical additives such as monoglycerides and some esters.

There are also hemicellulases that will degrade the linear polymers by endo (random) or exo (from one end) action. The endo action will reduce the average polymer size and create a large number of smaller fragments with a substantial increase in hydration. This contributes very significantly to the texture and shelf-life of the product. The exo action also reduces the polymer size but releases mono- and dipentose saccharides. At low activity this is a noticeable effect by reducing the energy required to work the dough. At high activity, the result is a rapid increase in hydration and solubility and a very sticky dough is likely. In working with these concepts and to facilitate understanding of baking test results, the author has adopted the designation:

- Pentosanase I for the enzyme breaking cross-linkages and removing side branches.
- Pentosanase II for the enzyme depolymerizing by endo action.
- Pentosanase III for the enzyme depolymerizing by exo action.

Several enzyme companies offer hemicellulase/pentosanase enzyme preparations. These are usually fungal products and of extremely high activity. Individually these enzymes find use in the bakery mainly as components of prepared 'bread improvers'. None of the commercial products of this type is single activity materials, and most of them have at least two and many have all three of the component hemicellulase types in them. Each preparation will have a different ratio of the three types, and this will form the basis for a positive choice after they have been used in test baking. Examples of preparations (all from Biocatalysts) with strong levels of these three types of hemicellulase (pentosanase) are:

- Depol 356 (rich in type I, providing improved volume)
- Depol 351 (rich in type II, providing volume and texture)
- Depol 350 (rich in type III, providing volume and improved shelf-life).

The presence of these enzymes in regular fungal amylase preparations contributes to the understanding of the variations of performance and the preferences shown by the baker. Thus it is demonstrated by real baking practice that the selected application of the non-starch carbohydrases has positive benefit to all the criteria listed in Table 2.5.2. Examples of products where the side activities contribute to better performance than amylase alone are: (1) for extended shelf-life: Depol 360 (Biocatalysts); and (2) for extended shelf-life and improved texture: Depol 260 (Biocatalysts).

When there are approvals for the use of hemicellulases in the baking industries around the world we shall see fungal amylase preparations having very precise additional activities created by positively blending small quantities of the separate enzymes into the base amylases.

### 2.5.5.5 Processing for retarded or frozen doughs

The preparation of doughs intended to be chilled and used in batches over a period of time (retarded) or deep frozen for later thawing and baking demands special attention to the final texture and appearance of the products. Characteristically these products bake-out to a more dense structure and poorer texture than directly baked equivalents.

Improvement to these characteristics can be obtained by selecting an enzyme mixture that will reduce any risk of starch solubilization and subsequent retrogradation, that will improve the hydration stability of the dough, and support gas expansion after the period of chilling or freezing.

The mechanisms by which certain enzyme preparations do this are not defined. It is well established that some preparations can do this. Preparations in the Depol 276 range (Biocatalysts) are examples of suitable formulations.

## 2.5.6 PROTEINASES AND PEPTIDASES

The very considerable differences in dough and final product characteristics that are presented by the range of baked goods produced in the world clearly require that the common raw materials be modified and processed differently. The selection of wheat variety to focus on biscuit (cookie) or bread and buns production has a considerable part in this diversity. In addition, as has been presented above, the application of different enzymes can make significant differences to how the dough develops, is mechanically handled, and finally bakes out. The most important functional component of wheat flour is the protein. The functionality is largely attributed to the insoluble glutenins and gliadins, and

the most common discussion takes place around the general term 'wheat gluten'. As gluten hydrates in the dough mixing stage, it develops the changes in its cross-linking and total spatial structures that provide the unique viscoelastic properties that make wheat the main baking raw material.

These properties are clearly linked to changes in the character of the carbohydrates although the biochemical detail of the interlinking effects remains to be determined. The effects of different proteinases can be assessed by tests and trial baking. There will be influences from the hydrolytic preference of a particular enzyme for certain amino acid pairs, the pH range of its activity, and the thermal deactivation temperature within the baking programme. If the proteinase is an endo-enzyme with a wide selection of bonds that it will attack, and is operating at its preferred pH, then there will be a dramatic reduction in the size of the protein molecules, and the small peptide products will be more soluble. This extreme action would result in a complete loss of gluten viscoelasticity, and be of no useful benefit for baking. It could be brought under some control by selecting a very small dose and allowing a very short time for it to act before the baking temperature reached the deactivation value. The exo-proteinase, however active, will tend to reduce the polymer length relatively slowly, and so cause only modest changes to the functional properties of the hydrated gluten.

Peptidases are observed to act either at the carboxy or the amino ends of amino acids. There are usually mixtures of both types in commercial peptidase preparations although the proportions will vary from preparation to preparation. It is also noted that there are both endo and exo-peptidases.

### 2.5.6.1 Proteinase activity determination

Historically developed methods of assay for proteinase activity remain very much the industry standards. For baking, the hydrolysis of haemoglobin and the measurement of products that are soluble in strong trichloracetic acid (TCA) is the basic method. The action of an endo-proteinase may produce almost no increase in TCA solubles, yet it can have a very significant effect on dough character. Similarly, any proteinase that has the ability to vigorously solubilize haemoglobin does not necessarily do the same for gluten if its bond preferences are not present in gluten at a significant frequency. These principal variations mean that for any given stated value of haemoglobin activity, there will be a very diverse range of effects from each enzyme preparation used, even at the identical haemoglobin unit concentration.

### 2.5.6.2 Peptidase activity determination

It is usual to find that peptidases are specified in units of either amino or carboxy peptidase activity that refer to the hydrolysis of a specified synthetic peptide. The actual action in a dough cannot be predicted from this information. There will be changes in the rate of attack and access to the preferred bond pairs because the enzyme is working on a complex natural substrate. The presence in wheat gluten of the preferred bond pairs is also not usually established. The decision to investigate the use of proteinases in a particular baking formula depends on good information that indicates there is a need to modify the protein. If the flour is to be used for biscuits (cookies) or wafers and waffles then a reduction in elasticity in the dough is essential. The application of a bacterial proteinase is indicated. Where there is a requirement to achieve good product without the use of sulphur dioxide, good results can be obtained using bacterial proteinases and even better overall results may be obtained when a blend of bacterial and fungal proteinases together with hemicellulase is used.

For most bread production, there is little reason to introduce additional proteinase activity. If the gluten is a little strong, or there is a need for a very crisp and well-coloured crust and an improved flavour, then the addition of a carefully controlled dose of fungal proteinase with high peptidase content can be beneficial. The peptidase component is targeted at the soluble proteins of the flour so that small peptides and amino acids shall be released. Examples of enzymes suitable for increasing the relaxation of doughs and adding proof stability are Promod 357 and Promod 388 (Biocatalysts).

## 2.5.7 PRACTICAL INTERPRETATION OF ENZYME PERFORMANCE

The baking industry has adopted a series of extremely practical and well-interpreted gross tests to be performed directly on doughs. These are based on the use of mechanical means to apply stresses to the dough and to plot changes that occur so that they may be compared and interpreted. Originally designed to evaluate the likely performance of wheat flours for different baking purposes, these methods have been adapted to assist in the interpretation of the effects of adding various substances including enzymes. When using these tests it is very important to ensure that all aspects of the dough composition are constant for a series of comparative evaluations except the single parameter being evaluated.

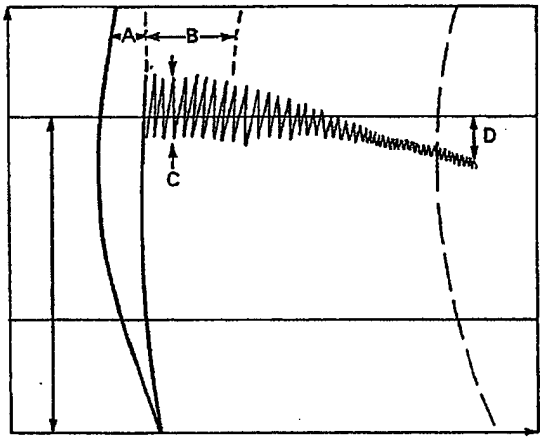


**Figure 2.5.1** The classical farinogram

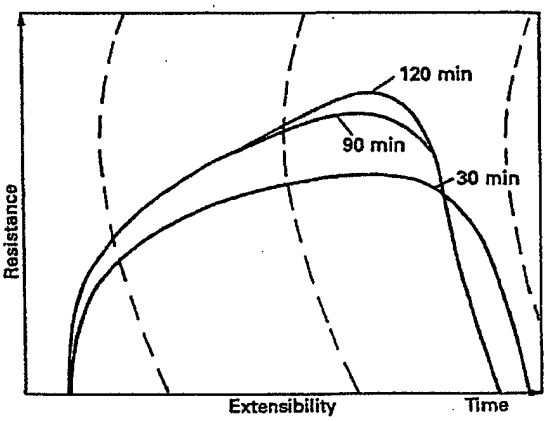


**Figure 2.5.2** Extensograms for quality bread flour at three dough development times

### 2.5.7.1 Farinograph

This measures the force required to turn the blades of a mixer at a constant speed. The recorded information is provided on a chart (farinogram) which provides the following information (Figure 2.5.1). By preparing the dough to give a constant initial resistance (usually set between 450 and 500 FU), the amount of water required to achieve this value is a measure of the consistency of the dough. It reflects the quality of the handling character, the machinability, and the economic relationship between flour and water. The time (A) that it takes the dough to achieve the set value is an indicator for the hydration rate. The time (B) is the period from achieving the set value until it starts to fall below the set value. This is an indication of the stability of the dough and will be reflected in machinability and the practical operating times for production of doughs and their fermentation tolerance, which will be gas retention ability, texture, and crumb conformation in the product. The distance (C), about which the force initially oscillates, is used to indicate the strength of the dough. The greater this value the stronger the dough and the greater will be its stability. The fall in force (D) measured at a specific time after the initial standard force has been achieved is a further strength indicator. The greater the fall the weaker the dough.

Farinograms can be valuable to explore the influences of various enzymes on the dough characteristics. It is found that the application of hemicellulases can increase the proportion of water that will be required to obtain the initial force value. The addition of a hemicellulase that increases the values for B and C may be expected to improve the gas retention (a volume improvement potential) and the stability (a potential improvement to the bake out quality). The presence of an unsuitable amylase activity will be observed as an exceptional increase in the water to flour ratio, a lowering of the values of A, B and C and an increase in the value of D. Proteinase activity will affect all the values, and marked differences will occur depending on the type of proteinase applied. Extremely small levels of additional proteinase activity of fungal origin can be beneficial to dough and bread performance, but overdosing, or the use of an aggressive bacterial proteinase, will show marked effects on the farinogram with poor stability, strength, and stability.

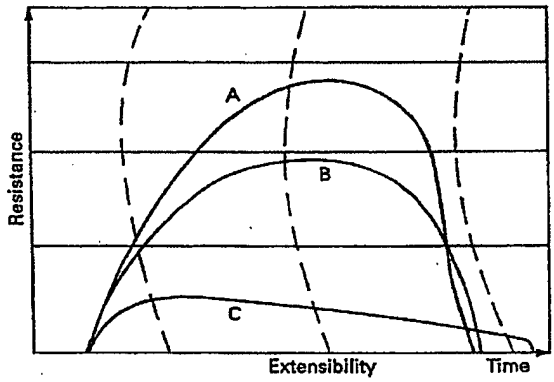


**Figure 2.5.3** Extensogram of typical flours. (A) Hard wheat (N. American) flour, (B) European bread flour and (C) European biscuit (cookie) flour

### 2.5.7.2 Extensograph

To evaluate the relative extensibility of doughs it is useful to apply this device which measures the force required to stretch and break a strand of dough drawn from a piece that is clamped at each end. This measure gives an indication of the strength and stability of the dough. It is mainly a measure of the gluten properties and in terms of enzymes it will be most affected by proteinases and peptidases. The result is a curve drawn on a chart (extensogram; Figure 2.5.2). It is usual to make measurements on samples from the same batch of dough after different periods of fermentation. If the force required increases with time, the dough is considered to have high strength.

The characteristics required of different wheat flours used to prepare different products are well demonstrated on this instrument (Figure 2.5.3). Curve A shows a strong Canadian wheat flour. Curve B shows a typical European baking wheat flour. Curve C shows the typical curve for a biscuit (cookie) flour.

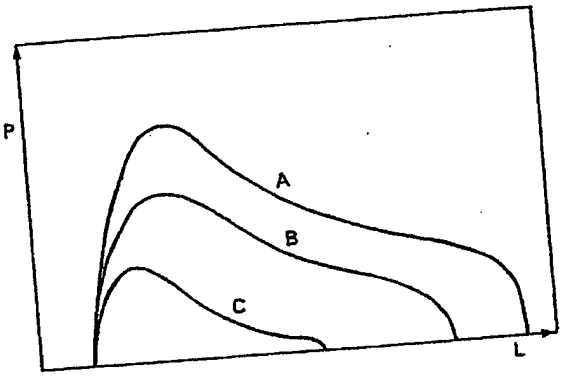


**Figure 2.5.4** Alveogram of typical flours. (A) Hard wheat (N. American) flour, (B) European bread flour and (C) European biscuit (cookie) flour

### 2.5.7.3 Alveograph

This instrument originally found use to measure the potential for volume development in the dough, and also gives some indication of the stability of the volume and shape of the baked product. By forcing air into a disc of dough, the pressure required to inflate and subsequently burst a bubble is measured over time. The more common use is to establish a predetermined bubble volume and then to cut off the air. Measuring the time and shape of the relaxation of the bubble as it loses the entrapped air gives valuable information about the gas retention properties of the dough. For evaluating doughs for biscuits (cookies), the charted information (alveogram) is related to the extension and elastic properties of the dough (Figure 2.5.4). The height is a measure of the resistance and the length indicates the relative relationship between extensibility and retained elasticity. The lower the elasticity while retaining good extensibility the better the dough will be for forming stable thin flat sheets from which to cut the biscuits. In order to use this instrument to evaluate the effects of added enzymes and other chemicals the reaction times should be extended, and also repeated measurements taken from the same batch of dough at different times.

## 2.5.8 THE CONTINUOUS ADD-ON ISSUE AND BREAD IMPROVERS

From the discussion so far it will be seen that there are a considerable number of potential materials that will be added variously to breads and other baked goods. These have been found and developed over many years, and each one is promoted for a particular effect. The general result has been that these are therefore brought into use without the removal of any others. This is not a universal situation but is sufficiently common to be addressed as an issue. The increased customer preference for fewer chemical additives in foods, and the growing interest to use enzymes for technical processing improvement, creates a potential area of conflict and also an area of confusion. The conflict arises because there is a risk of incompatibility between chemicals and enzymes.

### 2.5.8.1 Bromate replacement

Strong oxidizing agents such as potassium bromate can be seen to reduce the performance of added enzymes. The desire to find an alternative to bromate has encouraged a request that enzymes should be found that function in the same way and so be direct replacements. This has not been found to be the case.

The actions of bromate are not fully documented or agreed on, and this makes a serious problem for the enzyme supplier. The resolution has been to develop mixed enzyme formulations that actually deliver the required result by different molecular transformations within the dough to those likely to be performed by bromate. The product Combizyme 359 (Biocatalysts) is an example of this type of multi-enzyme formulation for bromate replacement. The confusions arise when there is more than one route by which these extra materials reach the dough.

### 2.5.8.2 Bread improvers

It is a widely used practice that the industrial bakery will purchase a separate preformulated concentrated package of ingredients that can be added to the basic dough recipe. These are prepared by different compan-

ies who market them in presentations that are designed for the production of particular finished goods, e.g. batch bread, sliced bread, brown/wholemeal bread, batch rolls, burger buns, morning goods, etc. These 'improvers' contain many different substances and usually include a selection of enzymes. Apart from the small amount of fungal amylase added by the flour miller to correct the base value, there need not be any other enzymes or chemicals except for those in the 'improver'. In order to achieve market penetration, however, millers may improve their product differentiation by adding amylases selected for their attractive side activities. If the regulations are approved, hemicellulases may also be included.

By using an 'improver' the baker may inadvertently be taking in a different set of enzymes together with chemicals that may compete with or inactivate enzymes. The 'improver' manufacturer will have run test baking trials to ensure that his formula performs as it should. It is advisable that additional trials be run using the actual flour from the baking customer. The baker may also have technical support that allows him to investigate the application of additional enzymes added directly to the mix. In this case it is important that these additions are evaluated in the complete system, including any 'improvers' the baker intends to use.

These precautions will avoid unhappy results that might arise from the compound addition of some enzyme types with consequent overdosing. Enzymes supplied to 'improver' manufacturers are usually of substantially higher activity than those intended for direct baking use (up to 50 times stronger in some cases). Intending users should note the recommended dose rates and application advice from the enzyme supplier in order to ensure that they compare products of similar potency.

## 2.5.9 OTHER GRADES OF WHEAT AND OTHER FLOURS

There are a multitude of different baking practices around the world and many of these do not use wheat, or they use wheats of qualities different from that for bread production.

**Table 2.5.3** Examples of products made from soft wheat

| | |
|---|---|
| Biscuits, cookies | Crackers |
| Wafers | Pretzels |
| Cakes of all types | Pastries |
| Waffles | Pancakes |
| Doughnuts | Noodles |
| Flat breads | Pie crusts |

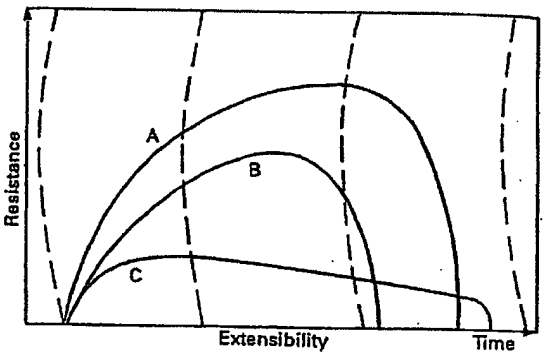


**Figure 2.5.5** Extensogram of addition of protease (Promod 223P) to wheat flour dough. (A) No enzyme addition (control), (B) low addition rate and (C) high addition rate

### 2.5.9.1 Soft wheat

There are very many applications of the soft wheat. Some of them are given in Table 2.5.3. For the group of products that includes biscuits, cookies, crackers, waffles, and pretzels, the most important characteristic of the dough is that it should have high extensibility and low elasticity. The product must retain shape, and often also an imprinted pattern. A second important feature is that highly hydrated gluten will lose a lot of water on baking and tend to produce a dry and readily cracked product. This must be avoided or controlled. The application of chemicals (e.g. sulphur dioxide) or proteinase enzymes to relax the dough and create the correct extension and loss of elasticity will increase the hydration potential rapidly. The rapidly spreading ban on the use of sulphur dioxide has increased the use of proteinases.

A typical application would be to use a selected bacterial proteinase. The selection will be for an enzyme with a thermal deactivation temperature that can be reached early in the baking stage. A more attractive enzyme would also have a limited number of bonds that it could break in the gluten molecules and so be effectively self-limiting. An example of this type of product is Promod 223 (Biocatalysts). The evidence for the performance potential may be readily obtained by applying the extensograph to test doughs. Figure 2.5.5 illustrates the typical extensogram for Promod 223.

Enzyme action to assist in the improvement of the final quality and to resist the tendency to cracking is also of value. This is provided by including some hemicellulase in the formulation where this is permitted. This acts to increase hydration without affecting the gluten. It also increases the carbohydrate–protein interaction giving a smoother finish.

An alternative is to select a fungal proteinase with suitable character that has an effective hemicellulase

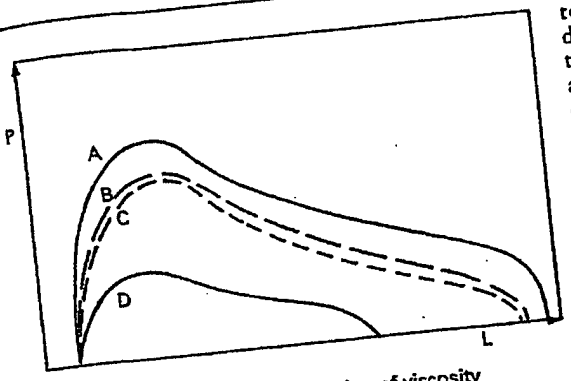


**Figure 2.5.6** Alveogram of addition of viscosity regulator (Depol 112P) to wafer dough, with continuous mixing. (A) Initial condition, (B) with enzyme at 15 min, (C) with enzyme at 360 min and (D) no enzyme at 360 min

side activity. An example of this type of preparation is Combizyme 366 (Biocatalysts).

Where the production of wafers and waffles uses a liquid batter that should be supplied to many baking stations via a pumped 'ring main', there is a requirement that the batter retains a constant viscosity over a very long time. The shear forces and slow enzyme actions in the batter are likely to cause the viscosity to fall over such a long time because of the depolymerization of glucan and the pentosan gums. If the batter is prepared from a flour that contains some appropriate additional enzyme to attack these molecules quickly, the mix can be made to a viscosity that will not readily change further.

The speciality amylase Depol 112 (Biocatalysts) is an example of this type of preparation. The use of the alveograph demonstrates the action of Depol 112 by comparing the alveograms of the same batch of batter over an extended time course (Figure 2.5.6).

**Table 2.5.4** Enzymes currently available for use in baking

| Enzyme type | Source | |
|---|---|---|
| Amylase | Bacterial | *Bacillus subtilis* |
| | | *Bacillus licheniformis* |
| | | Genetically manipulated organisms (GMOs) |
| | Fungal | *Aspergillus* – various spp. |
| | | *Rhizopus* – various spp. |
| Beta-glucanase | Bacterial | *Bacillus subtilis* |
| | Fungal | *Aspergillus* – various spp. |
| | | *Penicillium emersonii* |
| Catalase | Fungal | *Aspergillus niger* |
| Cellulase | Fungal | *Trichoderma* – various spp. |
| Glucose oxidase | Fungal | *Aspergillus niger* |
| Hemicellulase | Fungal | *Aspergillus* – various spp. |
| | | *Trichoderma* – various spp. |
| Lipase | Fungal | *Aspergillus niger* |
| | | *Candida cylindracea (rugosa)* |
| | | *Mucor* – various spp. |
| | | *Rhizopus* – various spp. |
| Lipoxygenase | Botanical | Soya flour |
| Pentosanase | Fungal | *Aspergillus* – various spp. |
| | | *Trichoderma* – various spp. |
| Peptidase | Fungal | *Aspergillus* – various spp. |
| | | *Rhizopus* – various spp. |
| Peroxidase | Botanical | Horse radish root |
| | Fungal | *Saccharomyces* – various spp. |
| Phospholipase | Animal | Pancreatic extracts |
| | Fungal | *Aspergillus* – various spp. |
| Proteinase | Bacterial | *Bacillus subtilis* |
| | Fungal | *Aspergillus* – various spp. |
| | | *Rhizopus* – various spp. |

### 2.5.9.2 Doughnuts

Flours for the production of doughnuts do not generally have additional enzymes other than the amylase in the flour. In attempts to reduce the fat content, however, it has been observed that the required structure and texture can be retained if a carefully selected blend of hemicellulases is included. The action is to break the cross-linkages and side branches from the long polymers so that they can contribute to the structure and substitute for the emulsified and free fats. An example of this type of preparation is Depol 365 (Biocatalysts). There is little evidence of the use of enzymes in the production of cakes, although the bleaching effects of lipoxygenases may become of importance if chlorination is to be replaced. To date there has been no substantial work carried out on the possible benefits of applying enzymes to the production of malt breads.

### 2.5.9.3 Durum wheat: noodles and pasta

The basic very hard nature of durum wheat, and the high protein content required to make good products, implies that there will be very little benefit from the carbohydrase enzymes. There are some small examples of the application of low levels of fungal proteinases to improve the surface gloss and to maintain a constant gluten strength with varying incoming flour strengths.

In the preparation of fast cooking and 'instant' products there is some interest for the use of a thermal tolerant bacterial amylase to rapidly solubilize the gelatinized starch on the surface after the initial cooking. This will reduce the sweet taste, and improve the separation of the product and avoid stickiness.

## 2.5.10 SUMMARY OF ENZYMES IN CURRENT USE FOR BAKING

Table 2.5.4 illustrates the range of enzymes in current use by the baking industry across a wide range of products and markets. Inclusion in the table does not confer

any certainty of regulatory approval for use in any particular country. The reader is advised to make careful enquiries of the authorities to establish the status of the enzyme type, source, and application.

## 2.5.11 POTENTIAL NEW ENZYMES FOR BAKING

Several enzymes with only a small initial presence in the baking world have been mentioned already. These will be summarized here together with a few others that are at the research stage. Very recently reported work by Chen and Hoseney (1995) describe work to uncover the agent responsible for 'sticky dough'. They have found a very small molecular weight material in the water-soluble fraction from sticky doughs that increases the stickiness when added to dough mixes that would not otherwise present stickiness. They provisionally identify this substance as ferulic acid esterified onto a carbohydrate chain of possibly 24 sugars. They point out that in wheat flour ferulic acid is esterified to pentosans and that they have not found any published reports of ferulic acid esterified to hexose sugars. These findings may well add support to the usefulness of certain pentosanases in the reduction of dough stickiness.

To create an oxidizing environment and so replace bromate with a similar chemical step there is interest for the application of glucose oxidase, peroxidase, and catalase. The enzymes can generate oxidation from glucose, or peroxides including hydrogen peroxide that might be added. Some technical problems have been noted so far. The main one is the rapid evolution and loss of the oxygen. Lipases are being evaluated to see if by being suitably selective it may be possible to generate mono- and diglycerides within the dough utilizing the fats and oils present. Lipoxygenases are being researched for both their potential protein oxidizing role (through lipid peroxides), bleaching effects, and as modifiers of lecithins to create internal emulsifiers. Enzymes in the group of disulphide reductase/disulphide isomerase are being investigated as possible contributors to controlled and effective gluten modification.

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# EXHIBIT D

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(54) **Bread improver and method of producing bread.**

(57) There is disclosed a bread improver which comprises glucose oxidase in combination with one or more hydrolases and, optionally, one or more oxidases. The improver may additionally comprise L-ascorbic acid. There is also disclosed a process for manufacturing bread which utilizes the same.

EP 0 468 731 A1

## Filed of the Invention

The present invention relates to a bread improver and, in particular, to a novel and safe bread improver not using potassium bromate.

The invention also relates to a novel method of manufacturing bread using the said novel bread improver.

## Prior Art

Yeast food has been invented in the U.S.A. in order to assist the fermentation of bread, and the classical Arkady-type formulation, consisting of $CaSO_4$ (24.93%), NaCl (24.93%), $NH_4Cl$ (9.38%), starch (40.49%) and $KBr0_3$ (0.27%), has also been developed in the country. Since then, various additives, including dough conditioners, have been developed to improve the volume, texture, etc. of bread.

Among such additives are included oxidizing agents usable as a bread improver, such as potassium bromate, potassium iodate and ammonium persulfate. Of these known bread improvers, potassium bromate has been used most widely.

In recent years, L-ascorbic acid has been used for health reasons, in place of potassium bromate. However, no satisfactory bread improvers have been known so far.

No hitherto known bread improvers can be effective in both shorter and longer fermentation time processes. In particular, known bread improvers for shorter fermentation time method are less effective and have a tendency to bring about a product of hard and crumbling texture. This tendency becomes marked with the lapse of time after their baking. Such bread also inevitably suffer from insufficiency of fermentation flavor.

## Problems to be Solved by the Invention

In Japan, the use of potassium bromate has been restricted by the government for the safety of food. Governments of other countries have also banned or have been considering to ban the use of the additive. It has therefore been strongly desired to develop a safe and effective bread improver usable in place of potassium bromate.

Also from the technical point of view, there has been a strong desire in this field of technology for an all-around bread improver which is not only highly effective in both shorter and longer fermentation time methods, but enables to manufacture bread having excellent flavor, texture, appearance and so on.

## Means Taken to Solve the Problems

It is therefore an object of the invention to provide a bread improver which can be free from the above disadvantages.

It is another object of the invention to provide a novel composition which can be used as an all-around bread improver for the manufacture of bread.

In order to achieve the above objects, the present inventors have conducted extensive investigations on a wide range of enzymes and, as a result, have completed the present invention.

The present invention is concerned with a bread improver comprising glucose oxidase (which may hereinafter be referred to as "GOD") in combination with oxidases other than glucose oxidase and hydrolases, and optionally with L-ascorbic acid, together with said combination of enzymes. The invention is also concerned with baking methods using the same.

GOD is an enzyme that specifically oxidize glucose to gluconic acid. The enzyme accelerates the oxidation of L-ascorbic acid and promotes the oxidative bonding of gluten to form tertiary structure in the dough.

As examples of oxidases other than GOD which can be used with advantage in the invention, mention may be made of catalase, lipoxidase, and the like. These oxidases can be used either individually or in combination of two or more. Catalase primarily decomposes hydrogen peroxide ($H_2O_2$) generated by GOD and hence promotes or complements the oxidative action of GOD. Lipoxidase is an enzyme that oxidizes unsaturated fatty acids, such as linoleic acid and linolenic acid. Carotene is also oxidized by this enzyme. The enzyme therefore supplements or promotes the oxidative action of GOD by accelerating the oxidation of the dough through oxidation of such unsaturated fatty acids, and consequently helps whitening and softening bread through oxidative bleaching of carotene contained in flour. However, in cases where these oxidases are used in large quantities, there tend to be resulted in undesirable tightening of the dough and also insufficient oven-spring and rough crumb grain of bread.

In the present invention, hydrolase can be used with advantage in order to complement or further improve the effects of GOD. Examples of preferable hydrolases include lipase, amylase, and the like. Hydrolases can

be used either individually or in combination of two or more. Lipase is an enzyme that hydrolyses triglycerides to glycerol and fatty acids. In the dough, lipids are hydrolysed by lipase, thereby forming surfactant which is capable of rectifying the drawbacks resulting from the use of GOD. That is, it suppresses the undesirable excessive toughening of the dough, improves the extensibility of the dough, gives a bigger and softer product and improves its flavor and keeping quality of bread. Amylase is an enzyme that hydrolyses starch to many kinds of sugars, such as dextrin, maltose, glucose, and the like. Such hydrolysates provide extensibility to the dough, improve the oven-spring and keeping quality of bread because of their water-holding property. In the baking industry, α-amylase has been used most widely.

In can be particularly advantageous to use GOD in combination with two or more of the above-described enzymes. As examples of particularly effective combination, mention may be made of the followings: A combination of GOD with lipase and/or amylase; a combination of GOD with lipase and/or amylase and with catalase and/or lipoxidase. Where necessary, these combinations can be additionally incorporated with L-ascorbic acid, and dried starch powder as a dispersant.

The amount of the above enzymes to be used varies depending on various factors, such as the enzyme's activity, kinds of bread, baking methods, e.g., fermentation time, and kinds of raw materials used. For example, in the case of a no-time dough method, enzymes can be used in quantities shown hereinbelow:

GOD (1,500 Units/g) is used preferably in an amount of ca. 1 to 200 ppm, more preferably ca. 10 to 100 ppm. When it is used less than the above lower limit, there will be resulted in an undesirably low oxidizing effect, whereas when it is used in an excessive amount, there will be resulted in an excessive tightening of the dough and a poor handling of the dough because of too much oxidation.

Catalase (50,000 Units/g) is used preferably in an amount of ca. 1 to 200 ppm, more preferably ca. 5 to 80 ppm. When it is used less than the above lower limit, there will be resulted in an undesirably low oxidizing effect, whereas when it is used in an excessive amount, there will be resulted in an excessive tightening of the dough and a poor handling of the dough.

Lipoxidase (lipoxidase-containing soybean powder, 500,000 Oriental Units/g) can be used preferably in an amount of ca. 200 to 20,000 ppm, more preferably ca. 500 to 2,000 ppm. When it is used less than the above lower limit, there will be resulted in an undesirably low oxidizing effect, whereas when it is used in an excessive amount, there will be resulted an undesirable deterioration in taste.

Lipase (60,000 Units/g)can be used preferably in an amount of ca. 50 to 1,000 ppm, more preferably from 100 to 600 ppm. When it is used less than the above lower limit, there will be resulted a low extensibility of dough and the desired softening of bread will be attained only insufficiently, whereas when it is used in an excessive amount, there will be resulted in an undesirably sticky and slack dough which could hardly be handled.

Amylase (10,000 Oriental Units/g) is used preferably in an amount of ca. 100 to 1,000 ppm, more preferably ca. 200 to 800 ppm. When it is used less than the above lower limit, there will be resulted a low extensibility of dough and the desired softening of bread will be attained only insufficiently, whereas when it is used in an excessive amount, there will be resulted in an undesirably sticky dough which could hardly be handled.

L-ascorbic acid enhances the oxidative effect with GOD and further strengthens the binding of the dough. The additive when used together with the above enzymes, further improves the baking properties. L-ascorbic acid is used preferably in an amount of ca. 5 to 500 ppm, more preferably ca. 20 to 200 ppm.

It should be noted that the above amounts are given just for illustration and the enzymes can be used in quantities not falling within the above ranges.

The units of enzyme activities employed herein are as follows:

| | |
|---|---|
| GOD activity: | One unit is defined as the activity of enzyme which catalyses the oxidation of one micromole of glucose per minute at 37°C and pH 5.1. |
| Catalase activity: | One unit is defined as the activity of enzyme which decomposes one micromole of hydrogen peroxide per minute at 25°C and pH 7.5. |
| Lipoxidase activity: | One unit is defined as the activity of enzyme which shows 0.001 of O.D. (at 234 nm) change per minute in substrate solution containing linoleic acid at 25°C and pH 9.0. |
| Lipase activity: | 10 units is defined as the activity of enzyme which liberates one micromole of fatty acids per minute when an olive oil emulsion is used as a substrate at 37°C and pH 6.0. |
| Amylase activity: | According to the Oriental method, wherein the viscosity decreasing time of a starch solution is measured by Ostwald Viscometer at 30°C and pH 5.0. (The amylase sample used in the present exhibited an activity of 10,000 Oriental units per gram). |

These enzymes can be prepared, e.g., by cultivating microorganisms or by extracting from plant or animal bodies. They can be used in either a purified or crude state. It is also possible to use cultured products (e.g., cell bodies of microorganisms, culture broths, culture filtrates, extracts, etc.) or extracts derived from animals

or plants, instead of enzymes. If desired, products obtained by concentrating, drying or diluting such cultured or extracted products can be used.

Further, any material that contains such enzymes can also be used, if desired, as it is. For example, soybean powder and various beans or bean products can be used in place of lipoxidase; and rice bran or its products can be used in place of lipase.

The bread improver of the present invention can be used for the manufacturing of bread in the same manner as in cases where commercial bread improvers are used. For example, it can be added into a mixer with other ingredients at the beginning of mixing process.

The bread improver can be utilized in any known baking methods, including no-time dough method, straight dough method, sponge and dough method, overnight dough method, low-temperature and long-time fermentation method, and frozen dough method. For example, in sponge and dough method, the improver can be added in two portions: one portion at sponge mixing, and the other portion at dough mixing. It is also possible to add to either sponge or dough. It can however be most preferable to add all the improver at sponge mixing.

The bread improver according to the invention can be an all-around improver. It can be used in both long time and short time baking method, and it can be highly suited for home uses, as well as for industrial uses.

According to the present invention, there can be obtained bread of a sufficient volume, which is satisfactory in the quality of the internal and external characteristics. Furthermore, the undesirable stickiness of the dough can be suppressed by the addition of the improver and the resulting dough can be handled quite smoothly. The effects of the improver is therefore marked and immediate from operation point of view.

The present invention will further be illustrated by examples. It should however be noted that the invention is by no means limited to these.

## Example 1

Bread improvers according to the invention were prepared by thoroughly admixing the ingredients set forth in Table 1.

Table 1

| Bread Improver | No. 1 | No. 2 | No. 3 | No. 4 | No. 5 |
|---|---|---|---|---|---|
| Glucose oxidase | 16.7% (60ppm) | 16.7% (60ppm) | 13.0% (30ppm) | 16.7% (60ppm) | 23.1% (120ppm) |
| Lipase | 83.3% (300ppm) | - | 43.5% (100ppm) | 55.5% (200ppm) | 57.7% (300ppm) |
| Amylase | - | 83.3% (300ppm) | 43.5% (100ppm) | 27.8% (100ppm) | 19.2% (100ppm) |
| Total | 100% | 100% | 100% | 100% | 100% |

Note: Figures in parentheses show theoretical concentrations of the ingredients (based on the weight of wheat flour).

## Example 2

Bread improvers according to the invention were prepared by thoroughly admixing the ingredients set forth in Table 2.

Table 2

| Bread Improver | No. 1 | No. 2 | No. 3 | No. 4 | No. 5 |
|---|---|---|---|---|---|
| Glucose oxidase | 3.0% (60ppm) | 3.0% (60ppm) | 1.5% (30ppm) | 3.0% (60ppm) | 6.0% (120ppm) |
| Lipase | 15.0% (300ppm) | - | 5.0% (100ppm) | 10.0% (200ppm) | 15.0% (300ppm) |
| Amylase | - | 15.0% (300ppm) | 5.0% (100ppm) | 5.0% (100ppm) | 5.0% (100ppm) |
| Dried starch (dispersant) | 82.0% | 82.0% | 88.5% | 82.0% | 74.0% |
| Total | 100% | 100% | 100% | 100% | 100% |

Note : Figures in parentheses show theoretical concentrations of the ingredients ( flour basis) in the case where the bread improver is used at a rate of 0.2% on flour weight.

Example 3

Bread improvers according to the invention were prepared by admixing the ingredients set forth in Table 3 (total weight of ingredients: 10 kg/lot) for a period of 7 minutes by use of a V-type mixer manufactured by Hosokawa Micron Co.

Table 3

| Bread Improver | No. 1 | No. 2 | No. 3 | No. 4 | No. 5 |
|---|---|---|---|---|---|
| L-ascorbic Acid | 2.5% (50ppm) | 2.5% (50ppm) | 5.0% (100ppm) | 5.0% (100ppm) | 5.0% (100ppm) |
| Glucose oxidase | 3.0% (60ppm) | 1.5% (30ppm) | 1.5% (30ppm) | 3.0% (60ppm) | 6.0% (60ppm) |
| Catalase | 3.0% (60ppm) | 1.5% (30ppm) | 1.5% (30ppm) | 3.0% (60ppm) | 3.0% (60ppm) |
| Soybean powder | - | 50.0% (1000ppm) | - | 50.0% (1000ppm) | 50.0% (1000ppm) |
| Lipase | - | - | 15.0% (300ppm) | 30.0% (600ppm) | 15.0% (300ppm) |
| Amylase | 30.0% (600ppm) | 15.0% (300ppm) | - | - | 15.0% (300ppm) |
| Dried starch (dispersant) | 61.5% | 29.5% | 77.0% | 9.0% | 9.0% |
| Total | 100% | 100% | 100% | 100% | 100% |

Note: Figures in parentheses show theoretical concentrations of the ingredients (flour basis) in the case where the bread improver is used at a rate of 0.2% on flour weight.

Example 4

Each bread of Sample Nos. 0 to 15 in Table 5 was prepared in accordance with the formula and the processing set forth hereinbelow, whereby bread improver Nos. 1 to 5 prepared in Example 2 were used for Sample Nos. 1 to 5 by 0.2% dosage, respectively, and bread improver Nos. 6 to 15 were used for Sample Nos. 6 to 15 (controls), respectively. No bread improver (No. 0 in Table 4) was used for Sample No. 0 (control).

In Table 4 are shown the compositions of bread improver Nos. 6 to 15 (controls).

The results obtained are shown in Table 5.

| Formula | |
|---|---|
| Bread flour | 100 % |
| Sugar | 5 % |
| Salt | 2 % |
| Shortening | 4 % |
| Yeast | 3 % |
| Bread Improver | (*) |

Note *: Bread improvers containing GOD, lipase, amylase, etc. as shown in Table 2 or 4 were used at ratio shown therein.

| Procedure | |
|---|---|
| Mixing | $L_1M_3 \downarrow^{\text{Fat}} M_3H_{8+2}$ |
| Dough temperature | 30 °C |
| Floor time | 15 min |
| Dividing | 450 g |
| Intermediate proofing | 15 min |
| Final proofing (35°C, 90% R.H.) | Up to 1.5 cm above the pan |
| Baking | 200 °C, 20 min |

Table 4

| No. | GOD(ppm) | | | | Lipase(%) | | | Amylase(%) | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | 10 | 30 | 60 | 120 | 0.01 | 0.03 | 0.05 | 0.01 | 0.03 | 0.05 |
| 0 | | | | | | | | | | |
| 6 | ○ | | | | | | | | | |
| 7 | | ○ | | | | | | | | |
| 8 | | | ○ | | | | | | | |
| 9 | | | | ○ | | | | | | |
| 10 | | | | | ○ | | | | | |
| 11 | | | | | | ○ | | | | |
| 12 | | | | | | | ○ | | | |
| 13 | | | | | | | | ○ | | |
| 14 | | | | | | | | | ○ | |
| 15 | | | | | | | | | | ○ |

Table 5

| No. | Handling of Dough | Final Proofing (min) | Rating of Bread | | | | | | Overall Rating |
|---|---|---|---|---|---|---|---|---|---|
| | | | Height (cm) | Volume (ml) | Appearance (10) | Crumb Grain (10) | Flavor | Texture | |
| 0 | X Weak | 49 | 11.8 | 2130 | 6 | 6 | X Powdery Odor | X Hard | X |
| 1 | ○ | 48 | 12.6 | 2380 | 7.5 | 7.5 | ○ | ◎ | ○ |
| 2 | ○ | 47 | 12.4 | 2340 | $7^+$ | $7^+$ | △ | ○ | ○ |
| 3 | ○ | 47 | 12.2 | 2360 | 7.5 | $7^+$ | ○ | ○ | ○ |
| 4 | ◎ | 47 | 13.0 | 2450 | $8^-$ | $7.5^+$ | ◎ | ◎ | ◎ |
| 5 | ○ | 48 | 13.1 | 2480 | $8^-$ | 7.5 | ◎ | ◎ | ◎ |
| 6 | △ | 48 | 11.9 | 2120 | $6^+$ | 6.5 | X | △ | △ |
| 7 | △ | 47 | 11.9 | 2150 | 6.5 | $6.5^+$ | X | △ | △ |
| 8 | ○ | 45 | 12.0 | 2220 | $6.5^+$ | $7^-$ | X | △ | △ |
| 9 | △ Tight | 47 | 12.6 | 2310 | 7 | $7^-$ | X | △ | △ |
| 10 | △ | 46 | 12.1 | 2220 | $6.5^-$ | $7^-$ | ○ | ○ Soft | △ |
| 11 | △ | 48 | 11.9 | 2250 | 6.5 | $6.5^+$ | ◎ | ○ Soft | △ |
| 12 | X Loose | 49 | 11.8 | 2260 | 6.5 | $6^+$ | ◎ | △ Sticky | △ |
| 13 | △ | 48 | 12.0 | 2200 | $6.5^-$ | 6.5 | △ | △ | △ |
| 14 | ○ | 46 | 12.2 | 2260 | 6.5 | $6.5^+$ | △ | △ | △ |
| 15 | △ | 46 | 12.2 | 2280 | $6.5^+$ | $7^-$ | ○ | △ | △ |



EP 0 468 731 A1

It would be apparent from the results shown in Table 5 that bread improvers containing two enzymes (in particular, GOD and lipase) can be highly effective and that further improved results can be attained by using bread improvers containing the three enzymes (i.e., GOD, lipase and amylase). It would also be apparent that satisfactory results can be obtained by using bread improvers without chemical additives.

Example 5

Each bread of Sample Nos. 1 to 5 in Table 6 was prepared by using bread improver Nos. 1 to 5 prepared in Example 3, by dosage of 0.2%, respectively. The results obtained are shown in Table 6.

The formula and procedure employed in the above preparation were as follows:

Formula

| | |
|---|---|
| Bread flour | 100 % |
| Sugar | 5 % |
| Salt | 2 % |
| Shortening | 4 % |
| Yeast | 3 % |
| Bread improver | (*) |

Note * : Bread improvers prepared in Example 3.

| Procedure | Fat |
|---|---|
| Mixing | $L_1M_3 \downarrow M_3H_8$ |
| Dough temperature | 30 °C |
| Floor time | 10 min |
| | 450 g |
| Intermediate proofing | 15 min |
| Final proofing (35°C, 90% R.H.) | Up to 1.5 cm above the pan |
| Baking | 200 °C, 20 min |

For the purpose of comparison, bread Nos. 6 to 8 in Table 6 were prepared in the same manner as the above, using the following bread improver Nos. 6 to 8, respectively, except that the bread improvers having the formulae set forth below, respectively, were used by dosage of 0.1%. In the following formulae, the figures in the parentheses show the concentrations of the ingredients by flour basis.

No. 6: L-ascorbic acid 10% (100 ppm)
Dried wheat flour 90%

No. 7: L-ascorbic acid 10% (100 ppm)
GOD 3% (30 ppm)
Dried wheat flour 87%

No. 8: Commercial bread improver for no time dough method
(based on L-ascorbic acid)

Table 6

| Run No. | | Handling of Dough | Rating of Bread | | | | | | | | | Overall Rating |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | Final Proofing (min) | Height (cm) | Volume (ml) | Appearance (10) | Crumb Grain (10) | Flavor | Texture | Hardness of Bread* (g/cm²) | | |
| | | | | | | | | | | 1 Day after Baking | 3 days after Baking | |
| Sample | 1 | ◎ | 51 | 12.6 | 2430 | 7.5- | 7.5- | ○ | ○ | 29.8 | 47.4 | ◎ |
| | 2 | ◎ | 49 | 12.8 | 2420 | 7.5+ | 7.5+ | ○ | ◎ | 23.5 | 44.6 | ◎ |
| | 3 | ○ | 48 | 12.9 | 2400 | 7.5 | 7.5- | ◎ | ◎ | 22.3 | 42.5 | ◎ |
| | 4 | ○ | 47 | 13.0 | 2450 | 7.5+ | 8+ | ◎ | ◎ | 18.7 | 41.4 | ◎ |
| | 5 | ◎ | 47 | 13.5 | 2480 | 8+ | 8+ | ◎ | ◎ | 20.6 | 40.3 | ◎ |
| Control | 6 | X | 48 | 12.2 | 2300 | 7- | 7- | X | X | 33.0 | 63.9 | X |
| | 7 | X | 48 | 12.6 | 2360 | 7.5+ | 7.5 | △ | △ | 32.4 | 56.0 | △ |
| | 8 | ◎ | 48 | 12.3 | 2380 | 7+ | 7 | △ | △ | 31.2 | 53.3 | ○ |

Note

Rating: ◎ ... Excellent, ○ ... Good, △ ... Fair, X ... Poor

Hardness of Bread: Bread was stored at 20°C and its hardness was determined by Rheometer manufactured by Fudo Co., whereby the 2 cm-sliced bread was pressed down to 1 cm by circular plunger.



EP 0 468 731 A1

It would be apparent from the results shown in Table 6 that excellent results can be attained by using bread improvers containing GOD in combination with one or more oxidases and one or more hydrolases, and that such bread improvers can be more effective than the currently commercial bread improvers based on L-ascorbic acid.

Merits of the Invention

The bread improver according to the invention is based on novel compositions consisting of natural enzymes and, where optionally used, L-ascorbic acid. It can therefore be highly advantageous in safety.

In addition, the bread improver is applicable to any bread manufacturing processes and can be an all-around type additive highly effective not only in shorter fermentation time method but in longer fermentation time method.

The bread improver brings about a dough having excellent extensibility and makes it possible to manufacture bread which has not only bigger loaf volume but excellent other qualities, e.g., flavor, texture, keeping quality, etc.

## Claims

1. A bread improver which comprises glucose oxidase in combination with lipase and/or amylase.

2. A bread improver which comprises glucose oxidase in combination with catalase and/or lipoxidase, and with lipase and/or amylase.

3. A bread improver according to claim 1 or 2, which additionally comprises L-ascorbic acid and/or dried starch powder.

4. A process for manufacturing bread, which utilizes a bread improver according to any of the claims 1 to 3.

European Patent Office

# EUROPEAN SEARCH REPORT

Application Number

EP 91 30 6669

## DOCUMENTS CONSIDERED TO BE RELEVANT

| Category | Citation of document with indication, where appropriate, of relevant passages | Relevant to claim | CLASSIFICATION OF THE APPLICATION (Int. Cl.5) |
|---|---|---|---|
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The present search report has been drawn up for all claims

| Place of search | Date of completion of the search | Examiner |
|---|---|---|
| THE HAGUE | 04 NOVEMBER 1991 | BEVAN S.R. |

CATEGORY OF CITED DOCUMENTS

X : particularly relevant if taken alone
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# EXHIBIT E

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## (54) A method of improving the properties of dough and the quality of bread.

(57) The invention relates to a method of improving the properties of dough and the quality of bread by adding to the dough, dough ingredients, ingredient mixture or dough additives or additive mixture an enzyme preparation comprising hemicellulose and/or cellulose degrading enzymes and glucose oxidase, or sulphydryl oxidase and glucose oxidase, the enzyme preparation being preferably used in combination with lecithin. The enzyme preparation of the invention has an advantageous effect on the processability of the dough and the properties of the final bakery product. The combination of the enzyme preparation of the invention and lecithin can advantageously replace bromate conventionally used as a baking additive.

EP 0 338 452 A1

# A method of improving the properties of dough and the quality of bread

The invention relates to a method of improving the properties of flour dough and the quality of a finished bakery product, wherein an enzyme preparation comprising hemicellulose and/or cellulose degrading enzymes and glucose oxidase, or sulphydryl oxidase and glucose oxidase, is added to the flour or to the dough. The enzyme composition of the invention enables the use of weak flour, whereby the dough has not only a good process tolerance (advantageous rheological properties during the bread making process) but also a good oven spring and the final product will possess an improved grain structure and increased bread volume. The enzyme composition of the invention can partially or fully replace conventional emulsifiers used as baking additives. Furthermore, the enzyme composition can replace bromate used in bread as a baking additive, though accepted only in a few countries, especially when the enzyme composition is used in combination with a conventional emulsifier, lecithin.

Cellulases/hemicellulases cleave non-starch polysaccharides contained in flour. This affects the water retention and water binding capacity, viscosity, and proofing (rising) capacity of the dough as well as the texture, aroma, taste and freshness of the bread.

Generally speaking, the use of cellulases/hemicellulases gives an improved oven spring to the dough and an improved bread volume, grain structure and anti-staling properties to the finished bakery product. However, the dough may become too slack and stickier, which may cause problems. It is thereby necessary to use dosages too low for an optimum baking result to be achieved, so that the enzymes in question cannot be utilized to the full extent. At low dose levels, cellulases/hemicellulases make the mechanical handling of the dough easier whereas the effect of cellulases/hemicellulases on the process tolerance, for instance, may be insufficient when used alone, wherefore emulsifiers have to be used as additives.

It has been found that the addition of glucose oxidase (GO) and sulphydryl oxidase (SHX) strengthens the dough. Flour having a low protein content is usually classified as weak. The gluten of weak flour (the extensible, rubbery mass formed when mixing flour with water) is very extensible under stress but does not return to its original dimensions when the stress is removed. Flour with a high protein content is classified as strong. The gluten of strong flour is less extensible than that of weak flour. It is more resistant to mixing.

Strong flour is often preferred for baking purposes, since the rheological and handling properties of a dough prepared from such flour are superior to those obtained with weak flour. In addition, the shape and texture of a bakery product prepared from strong flour are remarkably better as compared with weak flour.

A dough prepared from strong flour is also more stable as compared with that prepared from weak flour. This is one of the most important - if not the most important - properties in view of the baking process.

The stability of dough (process tolerance) can be improved by glucose oxidase and sulphydryl oxidase; however, the bread volume of the product obtained with these enzymes is not generally sufficiently good and the texture is not sufficiently good (velvety).

In addition to those mentioned above, enzymes affecting baking further include amylases and pro teases. Amylases produce sugars for yeast food (from damaged starch, for instance). Alpha-amylase breaks down such starch into dextrines which are further broken down by beta-amylases into maltose. Due to this, an increased amount of gas is produced by the yeast, which increases the bread volume. At the same time, the increased formation of dextrines and maltose improves the crust colour, aroma and taste of the final product. Furthermore, alpha-amylase retards the chemical ageing of bread (staling of the bread crumb). Protease, in turn, break down flour proteins, resulting in a more stretchy dough. The dough "matures" more rapidly whereby the need of mixing and the fermentation times of the dough can be decreased; due to the better baking properties, the gas retention of the dough, and the volume and grain structure of the bread are improved.

It has been known for a long time to use so called bread improvers in the preparation of dough. The function of such bread improvers, including emulsifiers, unspecific oxidants (such as ascorbic acid (dehydroascorbic acid), potassium bromate, peroxides, iodates, etc.) etc., is to form inter-protein bonds which strengthen the dough.

Emulsifiers used in baking have many effects, such as retardation of chemical ageing, strengthening of gluten and an even emulsification of fat through the dough. Conventional emulsifiers used in baking include monoglycerides, diacetyl tartaric acid esters of mono- and diglycerides of fatty acids, and lecithins. Lecithin used in baking is normally obtained from soya. Lecithin may be in many different product forms, such as raw lecithin, de-oiled lecithin, or a carrier spray-dried lecithin, fractionated lecithin, chemically modified and enzymatically modified lecithin. Lecithin is a mixture of different phospholipides, the composition of which is

variable. Furthermore, the different product types and commercial products behave in different ways in baking applications. Normally the lecithin content of commercial products is specified as acetone insoluble material (AI). Following commercial product examples from Lucas Meyer, Hamburg, Germany, illustrate the range of products: Emulpur N (de-oiled), phospholipide content min 95%; Lecimulthin M-035 (spray-dried), phospholipide content appr. 28.0%. In addition to its emulsifying effect, lecithin improves the baking properties of the other baking ingredients, increases bread volume, improves anti-staling properties and has a favourable effect on the crumb and crust texture.

Many commonly used bread improvers have disadvantageous effects; in particular, they may have negative organoleptic effects on the final bakery product. On the other hand, the use of bromate, e.g., is not accepted in many countries.

From the consumer's point of view, it is advantageous to minimize the use of the above-mentioned chemical additives.

U.S. Patent Specification 2,783,150 discloses a method of treating flours with glucose oxidase enzyme for improving the dough formation and baking properties. This results in improved dough strength, improved dough handling properties, and improved texture and appearance of the baked product. The use of glucose oxidase in combination with ascorbic acid is recited as particularly advantageous.

Japanese Patent Specification 5701/1968 discloses a method of improving the quality of bread by the addition of an enzyme composition containing cellulase and/or hemicellulase to the dough. It is empha sized in the patent specification that the addition of this enzyme composition causes decomposition of insoluble fibrous components contained in flour, such as cellulose and pentosan which as such would considerably deteriorate the quality of bread by rendering the dough non-homogeneous and by preventing the formation of gluten. It is recited that the bread product so obtained has an increased volume, more uniform grain structure and slower ageing during storage.

U.S. Patent Application 136,003, filed in December 1987, describes the use of an enzyme preparation containing glucose oxidase and microbiological sulphydryl oxidase for increasing the strength of a dough prepared from flour, water and yeast. Such an enzyme preparation is recited to improve the rheological properties of the dough and, in particular, to improve the stability of the dough.

The combination of glucose oxidase and sulphydryl oxidase has also been shown to dry the surface of dough, which improves the machinability of the dough.

It has now been unexpectedly found that the combined use of hemicellulase/cellulase and glucose oxidase enzymes, or glucose oxidase and sulphydryl oxidase enzymes has a complementary synergistic effect, so that the processability and process tolerance, oven spring, volume and texture are clearly better than what could be expected when using each one of these enzymes alone.

The invention relates to a method of improving the rheological properties of flour dough and the properties of the final bakery product by adding to the dough an effective amount of an enzyme preparation containing hemicellulase and/or cellulase and glucose oxidase, or glucose oxidase and sulphydryl oxidase. By the use of this enzyme composition, a dough prepared from weak flour will have the typical advantageous properties of a dough prepared from strong flour (advantageous rheological properties and "good gluten properties", handling properties and tolerance in a mechanized industrial bread making process) while the final bakery product keeps its desired shape, has good volume, good grain structure and good organoleptic properties. The enzyme composition of the invention can also either partially or fully replace conventional bread improvers classified as additives (e.g. emulsifiers). The surface of a dough containing the enzyme preparation of the invention remains dry, which is an important factor in industrial processes.

The dough is prepared by mixing together flour, water, yeast, the enzyme composition of the invention and other possible ingredients and additives. The enzyme preparation can be added together with any dough ingredient or ingredient mixture or any additive or additive mixture, except strong chemicals which inactivate the enzymes. The dough can be prepared by any dough preparation process common in the baking industry, such as a normal straight dough process, a sour dough process, the Chorleywood Bread Process, and the Sponge and Dough process. Wheat flour is preferably used but it is also possible to use, e.g., rye flours and other flours and their mixtures. The enzyme preparation of the invention can also be used in the preparation of dry grain products, such as ryecrisp and rusk.

The enzyme preparation comprises about 0-50,000 units, preferably 10-10,000 units of hemicellulolytic activity (calculated as xylanase units); about 0-50,000 units, preferably 10-10,000 units of cellulolytic activity (calculated as carboxymethyl cellulase units); about 5-2,500, preferably 35-1,000 units of glucose oxidase; and about 0-800, preferably 0-300 units of sulphydryl oxidase calculated per kg of flour (the enzyme units will be defined later). The preferred amounts of enzymes depend on the process used, the process conditions, and the ingredients. An example of an enzyme preparation useful in direct baking would be as

follows: 300 units of hemicellulase, 100 units of cellulase, 300 units of glucose oxidase, and 1 unit of sulphydryl oxidase per kg of flours. Enzyme preparations useful in Chorleywood baking include a preparation containing about 2,000 units of hemicellulase, about 700 units of cellulase, about 650 units of glucose oxidase, and about 2.5 units of sulphydryl oxidase.

Any method known from the prior art can be used in the preparation of the enzymes. Hemicellulolytic and cellulolytic enzymes can be prepared microbiologically by means of fungi or bacteria, e.g., molds belonging to the Trichoderma, Aspergillus or Penicillium genus, in a manner known per se. Sulphydryl oxidase and glucose oxidase can be prepared microbiologically by means of fungi and bacteria, e.g., molds belonging to the Aspergillus or Penicillium genus.

The hemicellulolytic and cellulolytic activities of the enzyme preparations of the invention are defined as xylanase(Xyl.), carboxymethyl cellulase(CMC) and/or filter paper(FP) activities.

The definitions of the different enzyme activities and the methods of defining the enzyme activities are set forth below:

Xylanase activity (Khan A.W. et al., Enzyme Microb. Technol. 8 (1986) 373-377):

1 ml of a suitably diluted enzyme solution in acetate buffer (0.05 M NaAc, pH 5.3) is tempered at 50° C. 1 ml of xylan substrate (1% xylan, 0.05 M NaAc, pH 5.3) is added. The sample is incubated for 30 min at 50° C. The reaction is stopped by adding 3 ml of DNS reagent (3,5-dinitrosalicylate), and the colour is developed by boiling the sample mixture for 5 min. The absorbance is measured at 540 nm. One enzyme unit liberates 1 micromole of reducing sugars per one minute under assay conditions, calculated as glucose.

Filter paper activity (Ghose T.K. et al., Symposium of Enzymatic Hydrolysis of Cellulose, Bailey M., Enari T.M., Linko M., Eds. (SITRA, Autanko, Finland, 1975), p. 111-136):

A piece of filter paper (Whatman 1, 50 mg) is added to 1 ml of acetate buffer (0.05 M NaAc, pH 4.8). 1 ml of suitably diluted enzyme solution is added. The solution is incubated for 1 h at 50° C. The reaction is stopped by adding 3 ml of DNS reagent, and the colour is developed and measured similarly as in the xylanase determination. One activity unit liberates 1 micromole of reducing sugars per one minute under assay conditions, calculated as glucose.

Carboxymethyl cellulase activity (Mandels M., Weber J., Adv. Chem Ser. 95 (1969) 391-413):

1 ml of suitably diluted enzyme solution in acetate buffer (0.05 M NaAc, pH 4.8) and 1 ml of CMC substrate (1% CMC, 0.05 M NaAc, pH 4.8) are mixed together. The solution is incubated for 10 min at 50° C. The reaction is stopped by adding 3 ml of DNS reagent. One enzyme unit liberates 1 micromole of reducing sugars calculated as glucose per one minute, under assay conditions.

Sulphydryl oxidase activity (Young J. and Nimmo I., Biochem. J. 130 (1972) 33):

One sulphydryl oxidase unit is equal to an enzyme amount required for depleting 1 micromole of $O_2$ per one minute from a test mixture containing 8 mmol of GSH (reduced glutathione) and 40 mmol of sodium acetate (pH 5.5) at 25° C.

Glucose oxidase activity (Scott D., J. Agr. Food. Chem. 1 (1953) 727):

3 units of glucose oxidase yields 1 ml of 0.05 N gluconic acid.

The enzyme preparation of the invention may contain cellulases and/or hemicellulases functioning both with endo- and exomechanisms. In addition to these enzyme activities, the enzyme preparation to be used according to the invention may contain substantial amounts e.g. of the following enzyme activities: beta-glucosidase, beta-xylosidase, acetyl esterase, arabinase, mannanase, galactomannanase, pectinase, alpha-arabinosidase, alpha-glucuronidase, alpha-amylase, beta-amylase, glucoamylase and protease.

Example 1 (pan bread, white bread dough)

Baking tests were carried out in which two different types of enzyme preparations containing hemicellulolytic and cellulolytic activity (preparations A and B), enzyme preparation containing glucose oxidase and sulphydryl oxidase (preparation C), and enzyme preparation of the invention containing cellulolytic and hemicellulolytic activity and glucose oxidase and sulphydryl oxidase (preparation D) were added to a pan bread dough.

The enzyme activities of the enzyme preparations to be tested appear from the following Table 1, whereby xylanase(Xyl.), carboxymethyl cellulase(CMC) and filter paper(FP) activities are descriptive of the hemicellulolytic and cellulolytic activity of the enzyme preparations (preparations A and B). Preparation C contains glucose oxidase and sulphydryl oxidase, and preparation D of the invention contains glu cose oxidase (GO) and sulphydryl oxidase (SHX) in addition to the above-mentioned cellulolytic and hemicellulolytic activities.

Table 1

| Enzymes to be tested | | | | | | |
|---|---|---|---|---|---|---|
| Preparation | Dosage mg/kg of flour | Enzyme activity U/kg of flour | | | | |
| | | Xyl. | CMC | FP | GO | SHX |
| A. Control 1 (cellulase + hemicellulase) | 1. 12.95 | 350 | 120 | 5 | - | - |
| | 2. 25.90 | 700 | 240 | 10 | - | - |
| | 3. 37.00 | 1,000 | 340 | 14 | - | - |
| | 4. 74.00 | 2,000 | 680 | 28 | - | - |
| B. Control 2 (cellulase + hemicellulase) | 1. 40 | 20 | 165 | 14 | - | - |
| | 2. 80 | 40 | 330 | 29 | - | - |
| | 3. 160 | 80 | 660 | 58 | - | - |
| C. Glucose oxidase + sulphydryl oxidase | 1. 0.8 | - | - | - | 100 | 0.4 |
| | 2. 2.4 | - | - | - | 300 | 1.2 |
| | 3. 4.8 | - | - | - | 600 | 2.4 |
| D. Combination: cellulase + hemicellulase + glucose oxidase + sulphydryl oxidase | 1. 37/2.5 | 1,000 | 340 | 14 | 320 | 1.3 |
| | 2. 37/5 | 1,000 | 340 | 14 | 645 | 2.6 |
| | 3. 74/2.5 | 2,000 | 680 | 28 | 320 | 1.3 |
| | 4. 74/5.0 | 2,000 | 680 | 28 | 645 | 2.6 |

Flour used in the test bakes possessed the following properties:

| | |
|---|---|
| Moisture (%) | 14.7 |
| Protein content (Kjeldahl) (%) | 11.3 |
| Concentration of damaged starch (Farrand units) | 28 |
| Alpha-amylase content (Farrand units) 2 | |
| Colour of flour | 3.3 |
| Falling number (5 g) | 218 |
| Water binding in 10 min (% on flour) | 58.6 |

Composition of the dough in the test bakes was as follows (amounts are percentages on the amount of flour):

| | |
|---|---|
| Flour | 100 |
| Yeast | 2.1 |
| Salt | 1.8 |
| Fat | 0.7 |
| Water | 58.6 |
| Ascorbic acid | 0.003 |
| Potassium bromate | 0.0045 |
| Enzyme additions (see Table 1) | |

Flour, salt, ascorbic acid and bromate were weighed and stored at constant temperature (21 °C) overnight. Each enzyme preparation was dissolved in water at a desired concentration before each test series. A dough was prepared by the Chorleywood Bread Process, whereby each dough batch contained 1,400 g of flour. The flour was first introduced into a mixing bowl, whereafter the other dry ingredients were added. The enzyme solution was dispersed through the dough water, and the resultant solution was added to the dough. The dough was prepared as follows: mixing (Morton Kopp mixing device, mixing speed 300 rev./min), scaling and first moulding, first proof (10 min), final moulding, final proof at 43 °C (proof height 10 cm), and baking at 230 °C for 25 min. Thereafter the loaves were allowed to cool, and they were stored overnight in a closed space at constant temperature (21 °C), whereafter the bread volume was determined by the rapeseed displacement, and other desired properties were determined.

The obtained results appear from Table 2 for the enzyme preparations A, B, C and D. The following properties are given in the different columns:

m = amount of added enzyme preparation (mg/kg of flour)
K = dough consistency (subjective assessment)
t = proof time (min) (= time taken by the dough to reach a height of 10 cm in the pan)
h = oven spring (cm) (= difference between the heights of unbaked and final baked loaf)
V = bread volume (ml) determined by rapeseed displacement
$\Delta V$ = change (%) in bread volume with respect to control
R = crumb score (from 1 to 10, the greater the value, the better the structure)

Each baking test was carried out as a parallel test in triplicate, and the evaluation of the loaves is given as the mean value of the results obtained for 3x4 loaves (same enzyme, same concentration).

Table 2

| Enzyme preparation | m | K | t | h | V | ΔV | R |
|---|---|---|---|---|---|---|---|
| A (comparison) | Control | good | 50 | 1.8 | 1349 | - | 8 |
| | 12.95 | good | 49 | 2.0 | 1363 | +1.0 | 8 |
| | 25.9 | good | 50 | 2.0 | 1375 | +1.9 | 8 |
| | 37.0 | good | 50 | 2.2 | 1415 | +4.9 | 7.3 |
| | 74.0 | very good | 51 | 2.3 | 1434 | +6.3 | 7.6 |
| B (comparison) | Control | good | 50 | 1.8 | 1349 | - | 8 |
| | 40 | smooth* | 50 | 2.2 | 1399 | +3.7 | 8.3 |
| | 80 | smooth* | 49 | 2.0 | 1388 | +2.7 | 8.3 |
| | 160 | very good | 50 | 2.2 | 1424 | +5.6 | 8.6 |
| C (comparison) | Control | good | 47 | 1.6 | 1332 | - | 7.3 |
| | 0.8 | good | 47 | 1.5 | 1321 | -0.8 | 7.3 |
| | 2.4 | good | 47 | 1.5 | 1306 | -2.0 | 7.6 |
| | 4.8 | very good | 46 | 1.8 | 1334 | +0.2 | 7.6 |
| D (according to the invention) | Control | good | 47 | 1.6 | 1332 | - | 7.3 |
| | 39.5 | good | 45 | 2.5 | 1443 | +8.3 | 7.7 |
| | 42.0 | relaxed** | 47 | 2.3 | 1443 | +8.3 | 7.7 |
| | 76.5 | extensible*** | 46 | 2.1 | 1449 | +8.8 | 7.7 |
| | 79.0 | extensible*** | 45 | 2.5 | 1441 | +8.2 | 8.3 |

*) machinability of the dough improved
**) the dough becomes stretchy with time, i.e., the gluten properties are improved so that the dough is easier to handle
***) elastic, slacker dough

It appears from the results that the preparation D of the invention, containing cellulose and hemicellulose degrading enzymes and glucose oxidase and sulphydryl oxidase enzymes, improves the handling properties of the dough (improved relaxation and elasticity) as compared with the comparison preparations, which contain either cellulolytic and/or hemicellulolytic activity (preparations A and B) or glucose oxidase and sulphydryl oxidase (preparation C). In addition, the bread prepared according to the invention has improved oven spring, volume and texture.

Example 2 (hearth white bread)

Baking tests were carried out by adding enzyme preparations C and D described in Example 1 to a bread dough, of which the latter preparation was the enzyme composition of the invention while the former contained glucose oxidase and sulphydryl oxidase. The enzyme activities and dosage of the tested enzyme preparations were the same as in Example 1. The composition of the dough used was the same as that of the pan bread dough of Example 1, except that it contained less water (55.0% on the amount of flour). The ingredients were pre-treated similarly as in Example 1, and dough batches of 5,000 g and 2,500 g were prepared for enzyme preparation C and enzyme preparation D, respectively, using the Chorleywood Bread Process. The enzyme solution was dispersed through the dough water, and the water was introduced into a mixing bowl. Then the flour and other dry ingredients were added. The dough was prepared as follows: mixing (Tweedy 35 mixing apparatus, 450 rev/min), scaling, first moulding, first proofing (6 min), second moulding, final proofing at 40 °C (proof times 50, 70 and 90 min) at 70% humidity and baking at 244 °C for 25 min. Then the loaves were allowed to cool, and they were stored overnight in a closed space at constant temperature (21 °C), whereafter bread volume was determined by rapeseed displacement, and the height and width of the bread were measured. Further, change (%) in bread volume was determined as compared with the control. The results appear from the following Table 3.

Table 3

| Enzyme prep. | Dosage (mg/kg) | Mixing time (s) | Vol.(ml) + vol. change (%) in rel. to control with different proof times* | | | Height (cm) | | | Width (cm) | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | 50 | 70 | 90 | 50 | 70 | 90 | 50 | 70 | 90 |
| C | Control | 93 | 1121 | 1110 | 1093 | 8.0 | 7.2 | 6.8 | 10.9 | 11.9 | 12.2 |
| | 0.8 | 102 | 1161+3.6 | 1071-3.6 | 1222+11.8 | 8.2 | 7.5 | 7.3 | 10.7 | 10.7 | 12.3 |
| | 2.4 | 98 | 1112− 0.8 | 1160+4.5 | 1214+11.1 | 8.5 | 8.6 | 7.3 | 10.3 | 10.5 | 12.5 |
| | 4.8 | 101 | 1143+2.0 | 1101-0.8 | 1244+13.8 | 8.2 | 8.1 | 7.8 | 10.4 | 10.4 | 12.1 |
| D | Control | 96 | 1164 | 1108 | 1168 | 8.5 | 7.8 | 6.8 | 10.9 | 11.5 | 13.0 |
| | 39.5 | 107 | 1295+11.3 | 1482+33.8 | 1355+16.0 | 7.9 | 7.6 | 6.6 | 11.7 | 12.9 | 13.8 |
| | 42.0 | 105 | 1286+10.5 | 1509+36.2 | 1425+22.0 | 7.6 | 8.2 | 6.5 | 11.6 | 12.5 | 13.5 |
| | 76.5 | 108 | 1249+7.3 | 1508+36.1 | 1558+33.4 | 7.6 | 8.0 | 7.5 | 11.3 | 12.8 | 13.9 |
| | 79.0 | 104 | 1300+11.7 | 1505+35.8 | 1529+30.9 | 7.8 | 8.2 | 9.3 | 12.1 | 12.5 | 13.3 |

* Proof times used were 50, 70 and 90 min.

It appears from the results that the effect of the enzyme composition preparation D of the invention on bread volume, for instance, is more favourable than that of the preparation C containing glucose oxidase and sulphydryl oxidase. In addition, bread prepared according to the invention maintained its shape even with long proof times whereas the control loaves showed a tendency to "flatten out".

Example 3 (hearth white bread)

In addition to those mentioned above, baking tests were carried out to study the replacement of emulsifiers used in bread improvers and classified as additives with enzyme preparations of the invention containing cellulolytic and/or hemicellulolytic enzyme activity and glucose oxidase. The analysis of the flour used in the baking trials gave the following results: moisture 14.8%, falling number 262, colour 3.7, gluten 26.0%, ash 0.77% (on dry basis), and swelling number 20 (ascorbic acid 15 ppm). The enzyme activities of the used enzyme preparations of the invention are shown in the following Table 4.

Table 4

| Preparation | Dosage mg/kg of flour | Enzyme activity U/kg of flour | | | |
|---|---|---|---|---|---|
| | | Xyl. | CMC | FP | GO |
| 1 | 6 | 100 | 34 | 1.4 | 260 |
| 2 | 10 | 200 | 68 | 2.8 | 260 |
| 3 | 17 | 400 | 136 | 5.6 | 260 |
| 4 | 21 | 500 | 170 | 7.0 | 260 |
| 5 | 8 | 100 | 34 | 1.4 | 530 |
| 6 | 12 | 200 | 68 | 2.8 | 530 |
| 7 | 19 | 400 | 136 | 5.6 | 530 |
| 8 | 23 | 500 | 170 | 7.0 | 530 |
| 9 | 12 | 100 | 34 | 1.4 | 1050 |
| 10 | 23 | 400 | 136 | 5.6 | 1050 |

The bread improver used in the tests contained bread improver base and 8% emulsifier (diacetyl tartaric acid esters of the mono- and diglycerides of fatty acids), and its analysis gave the following results:

| | |
|---|---|
| Alpha-amylase | 12 U/g |
| Xylanase | 18 U/g |
| CMC | 5 U/g |
| FP | 2 U/g |
| Ascorbic acid | 0.9 mg/g |
| Fat | 38% by weight |

In the test, the emulsifier of the bread improver (diacetyl tartaric acid esters of the mono-and diglycerides of fatty acids) was replaced with the enzyme preparation of the invention by adding it to the dough together with the improver base.

The baking conditions were as follows:

1) Formula

| | |
|---|---|
| Wheat flour, medium coarse (g) | 1700 |
| Yeast (g) | 50 |
| Salt (g) | 28 |
| Water (g) | 1000 |

2) Process

| | |
|---|---|
| Mixing | 6 min |
| Dough temperature | 27° C |
| Floor time 1 | 45 min |
| Floor time 2 | - |
| Scaling weight | 400 g |
| Transfer into pans | - |
| Proof | 40-45 min |
| Baking | 20 min/220° C |

The amounts of the added enzyme, bread improver and improver base appear from the following Table 5 showing the test results. 1.94% of the improver base was added to all doughs prepared with the enzyme com position of the invention. In each bake, a dough containing 2% of bread improver and a zero dough with no additives were used as a control.

The consistency of the doughs was measured by means of a pharinograph after kneading and proofing. The loaves were also measured for their height, width, specific volume, and softness.

Table 5

| Baking results | | | | | | | |
|---|---|---|---|---|---|---|---|
| Sample | Bread improver (g/kg) | Improv. base (g/kg) | Dough consistency (FU) | | Bread height/width | Specific bread vol. (l/kg) | Bread softness (penetrometer units) |
| | | | after mixing | after proofing | | | |
| No additives | - | - | 375 | 328 | 54 | 3.85 | 76 |
| Bread improver | 20 | - | 358 | 305 | 59 | 4.64 | 117 |
| 1 | - | 19.4 | - | - | 65 | 4.47 | 102 |
| 2 | - | 19.4 | - | - | 60 | 4.37 | 97 |
| 3 | - | 19.4 | - | - | 58 | 4.39 | 106 |
| 4 | - | 19.4 | - | - | 60 | 4.61 | 111 |
| 5 | - | 19.4 | 390 | 350 | 59 | 4.49 | 109 |
| 6 | - | 19.4 | 410 | 350 | 61 | 4.90 | 116 |
| 7 | - | 19.4 | 400 | 320 | 59 | 5.06 | 120 |
| 8 | - | 19.4 | 400 | 330 | 57 | 4.83 | 113 |
| 9 | - | 19.4 | 380 | 330 | 62 | 4.24 | 102 |
| 10 | - | 19.4 | 380 | 330 | 59 | 4.24 | 93 |

The enzyme composition of the invention made the dough harder than the bread improver, and it increased the mixing resistance of the dough and improved its proof tolerance as compared with the bread improver.

By means of the enzyme composition of the invention, white wheat bread could obtain a specific volume equal to or greater than that obtained by the bread improver. With the enzyme addition, the specific volume of the bread was at best about 9% greater than the specific volume of a corresponding bread containing bread improver and 31% greater than the volume of a product prepared without additives.

Loaves prepared with the enzyme composition of the invention were as soft as or slightly softer than those prepared with the bread improver and markedly softer than those prepared without additives. Loaves prepared with the bread improver showed a tendency to crack at the bottom.

Example 4

Bakery scale baking tests were carried out by adding to a white bread dough one enzyme composition which contained the three preparations with the enzyme activities mentioned in Table 6 (the qualities of the flour were identical with those in Example 3)

Table 6

| Enzyme preparation | Dosage mg/kg of flour | Added enzyme activities U/kg | | | | |
|---|---|---|---|---|---|---|
| | | Xyl. | FP | CMC | GO | SHX |
| 1. Cellulase/hemicellulase | 6.5 | 175 | 3 | 60 | | |
| 2. Fungal alpha-amylase ("Sal-Conc. 90 000", manuf. Shin Nihon, Japan | 5 | | | | | |
| 3. Glucose oxidase/sulphydryl oxidase | 4 | | | | 500 | 2 |

The object was to find out whether it was possible to replace the emulsifier and gluten additions used in baking with the enzyme composition in question.

Prior to the test bake, the enzyme composition was mixed with a small amount of wheat flour to form a so called baking pre-mixture. This pre-mixture was added at the beginning of dough mixing in such an amount that the enzymes were added at the dosages given in Table 6 per kg of flour. With this dosage, a white bread dough and a French bread dough were prepared. During the baking, the pre-mixture containing the enzyme additions was mixed with the flour prior to the addition of water.

The carrier in the pre-mixture may also consist of other ingredients than white flour, such as other flour, dry milk, sugar, fat or a mixture containing these ingredients. The possible carrier may also be a baking additive (such as an emulsifier) or an additive mixture containing baking ingredients and additives.

In addition to a normal baking test, a so called retarded baking test was carried out on the French bread dough, in which a dough piece in the form of a long loaf was kept in a refrigerator for 18 h, and the product was baked in the morning following the dough preparation. White bread was prepared using the straight dough process.

The ingredients and baking conditions were as follows:

1) Formula (amounts (g) calculated per one liquid litre of the dough)

| | French bread | White bread |
|---|---|---|
| Wheat flour | 1740 | see long loaf formula (no vegetable oil) |
| Gluten | 13 | |
| Yeast | 100 | |
| Salt | 28 | |
| Water | 1,000 | |
| Lecimax 2000 | 28 | |
| Vegetable oil | 19 | |
| | 2928 | |

2) Process

| | French bread | White bread |
|---|---|---|
| Mixing (min) | 17 | 12 (DIOSNA) |
| Temperature (°C) | 23 | 27 |
| Floor time (min) | 2-3 | approx. 30 |
| Moulding | First moulding | First moulding (BENIER) |
| Floor time (min) | 10 | 10 |
| Final moulding | Glimek | Glimek |
| Refrigerator (h) | 18 (part into direct baking) | direct baking |
| Proof (°C, %) | 32 | 30, 60%, 61 min |
| Baking | Stick oven (RADIONAL) | rotary grate 25 min (WERNER & PFLEIDERER) |

In baking trials with the enzyme additions, gluten and Lecimax 2000 were replaced with the defined enzyme-flour pre-mixture.

The baking results are shown in Table 7.

Table 7

| Product | Volume (ml) normal baking | Volume (ml) retarded baking | Softness (one day) |
|---|---|---|---|
| FRENCH BREAD | | | |
| Normal formula | 1080 | 1060 | |
| Enzyme comp. | 1250 | 1053 | |
| Difference % | (+16) | (± 0) | |
| WHITE BREAD | | | |
| Normal formula | 1855 | | 126 |
| Enzyme comp. | 1880 | | 108 |
| Difference % | (+1.5) | | (-17) |

| Sensory evaluation of the French bread and white bread bakes | | |
|---|---|---|
| | Normal formula | Enzyme composition |
| Dough after mixing | Rather weak | Velvety, strong |
| Dough handling properties | Slighty sticky | Dry surface, good machinability |
| Process tolerance of dough | Weak dough after proofing | Maintains well round profile at different process stages |
| Crust | Uneven texture and colour, flattish shape | Crust very uniform, round shape |
| Bread crumb | Slighty open grain | Uniform |

The results also from the bakery-scale test bake show that the white dough prepared with the addition of the enzyme composition was softer and more velvety after mixing than the dough prepared with the emulsifier and gluten addition. During moulding, the surface of the dough felt drier, which improved its machinability. During and after proofing, the dough pieces made of the dough with the enzyme additions had a greater height and exhibited a markedly better proof tolerance than the dough pieces made of the dough with the emulsifier and gluten addition. Differences observed during the baking process in the properties of the doughs manifested themselves in the final bakery products as improved appearance, i.e., the white bread and the French bread prepared with the enzyme additions had a more uniform surface and were more regularly round in shape. The test bake showed that by means of the enzyme composition the processability of the doughs could be improved and the final product had improved appearance and better crumb texture as compared with the bake using an emulsifier and gluten addition.

Example 5

Baking tests were carried out so as to find out whether it was possible to replace the bromate and/or diacetyl tartaric acid esters of the mono- and diglycerides of fatty acids (DATA esters) used as additives in baking with the enzyme composition of the invention in combination with lecithin. The following combinations (enzyme composition/lecithin) were used in the tests:

| | Combination A | Combination B |
|---|---|---|
| GO | 2.5 mg/kg | 2.5 mg/kg |
| Cell.-hemicell. | 25 " | 35 " |
| Fungal protease ("Fungal Protease", manuf. Biocon, Ireland) | 30 " | 30 " |
| Lecithin, Emulpur N | 0.4% | 0.4% |

The amount of the added enzyme composition is given in mg per kg of flour and the amount of added lecithin in % baked on flour.

The amounts of added cellulolytic and hemicellulolytic enzyme and glucose oxidase as enzyme activities per kg of flour were as follows:

| | Added enzyme activities U/kg | | | |
|---|---|---|---|---|
| | Xyl. | FP | CMC | GO |
| Combination A | 675 | 9.5 | 233 | 263 |
| Combination B | 945 | 13.3 | 326 | 263 |

In the test bakes, white pan bread was prepared using the Chorleywood Bread Process. The ingredients and baking conditions were as follows:

Basic formula:

| | % on the weight of flour |
|---|---|
| Flour | 100 |
| Compressed yeast | 2.5 |
| Salt | 1.8 |
| Water - determ. with a 10 min extrusion method | 57.5 g |
| Fat | 0.7 |
| Ascorbic acid | 0.003 |
| Potassium bromate | 0.0045 |

The alpha-amylases activity of the flour adjusted to 83 FU by adding fungal alpha-amylase.

Baking process:

| | |
|---|---|
| Mixing machine | Tweedy '35' |
| Mixing efficiency | 11 Wh/kg |
| Pressure | Atmospheric |
| Dough temperature | 30.5 ± 1 °C |
| Scaling | Manually into 908 g |
| First moulding | Into a ball with a conical moulder |
| First proof | 6 min at room temp. |
| Final moulding | "Four-piece" technique (R 9, W 15.5, P 0.25) |
| Pan size | 250 mm x 122 mm, height 125 mm |
| Shape | Lidded |
| Proof conditions | 43 °C, suitable humidity to prevent skinning |
| Proof height | 11 cm |
| Baking temperature | 244 °C |
| Type of oven | Gas-fired oven |
| Baking time | 30 min |
| Baking humidity | No steam injection |

With the formula described above, one prepared (1) a basic dough, (2) a basic dough without bromate, (3) a basic dough without bromate and DATA ester, (4) a basic dough without bromate and DATA ester but with the addition of the combination A of the enzyme composition of the invention and lecithin, and (5) a basic dough without bromate and DATA ester but with the addition of the combination B of the enzyme composition of the invention and lecithin. 5,000 g of flour was used in each dough batch.

No substantial differences were observed in the consistencies of the different doughs. The doughs were measured for the required mixing time (i.e. time required for the dough to consume 11 Wh/kg) and proof time, and the finished product for its loaf volume, Hunterlab Y-value (descriptive of the crumb colour, the higher the Y-value, the lighter the crumb colour), and the crumb score. The results are shown in Table 8.

Table 8

| | Mixing time (s) | Proof time (min) | Loaf volume (ml) | Hunterlab Y-value | Crumb score (max. 10) |
|---|---|---|---|---|---|
| (1) Basic dough | 120 | 51 | 3013 | 54.1 | 8.0 |
| (2) No bromate | 124 | 48 | 2914 | 53.5 | 5.5 |
| (3) No bromate, no DATA ester | 123 | 50 | 2594 | 50.5 | 2.0 |
| (4) No bromate, no DATA ester + (A) | 140 | 50 | 2925 | 49.3 | 4.0 |
| (5) No bromate, no DATA ester + (B) | 131 | 50 | 2953 | 51.2 | 5.0 |

The proof time was of the same order for all doughs (with the exception of dough (2)). As compared with the basic dough, the mixing time increased to some extent when the enzyme composition of the invention and lecithin were added to the dough. The addition of the enzyme composition and lecithin increased the loaf volume as compared with a product which did not contain bromate or DATA ester. No substantial differences were found in the crumb colour when comparing the product containing the enzyme composition of the invention and lecithin with a product prepared from the basic dough, which did not contain bromate and DATA ester. The crumb score was substantially better with the products (4) and (5) of the invention than with the product (3), which did not contain bromate and DATA ester. To sum up, it appears that the replacement of bromate and DATA ester with the enzyme composition of the invention and lecithin resulted in a marked improvement over products prepared from the basic dough containing no bromate and no DATA ester.

Example 6

Corresponding test bakes as above in Example 5 were carried out for replacing bromate and monoglycerides with the enzyme composition of the invention and lecithin except that the Sponge and

Dough technique was used as a baking process. The following combinations were used in the tests:

| | Combination | | |
|---|---|---|---|
| | C | D | E |
| GO | 1.0 mg/kg | 2 mg/kg | 3 mg/kg |
| Cell.-hemicell. | 15 " | 15 " | 30 " |
| Fungal protease ("Fungal Protease", manuf. Biocon Ireland) | 45 " | 45 " | 30 " |
| Fungal alpha-amyl. ("Sal-Conc. 90 000", manuf. Shin Nihon, Japan) | 5 " | 5 " | 5 " |
| Lecithin, Emulpur N | 0.4% | 0.4% | 0.4% |

The added amounts of the cellulolytic and hemicellulolytic enzymes and glucose oxidase as enzyme activities per kg of flour were as follows:

| | Added enzyme activities U/kg | | | |
|---|---|---|---|---|
| | Xyl. | FP | CMC | GO |
| Combination C | 405 | 5.7 | 140 | 105 |
| Combination D | 405 | 5.7 | 140 | 210 |
| Combination E | 810 | 11.4 | 280 | 315 |

Ingredients and baking conditions used in the baking tests were as follows:

White pan bread. preparation of basic dough

| Batch size (g) | (g) | Ingredients |
|---|---|---|
| | | Sponge |
| 700 | 2100 | White flour (protein content 11.72% determined per 14 % flour moisture) |
| 3 | 9 | "Arkady (RKD)" mineral yeast food, manuf. Cainfood Ind.; 2.8 g of bromate/kg |
| 25 | 75 | Compressed yeast |
| 420 | 1260 | Water |
| | | Dough |
| 300 | 900 | White flour (protein content 11.72% determined per 14% flour moisture) |

| | | |
|---|---|---|
| 60 | 180 | Sugar |
| 20 | 60 | Nonfat dry milk |
| 20 | 60 | Salt |
| 5 | 15 | Bread softener (monoglycerides) |
| 30 | 90 | All-purpose shortening |
| 180 | 540 | Water |
| 1763 | 5289 | Total dough weight |

| | | Process |
|---|---|---|
| | | Hobert A-200 mixer<br>McDuffee 20 Qt. dough bowl |
| | | Sponge: |
| 24-25<br>3.25 | 24-25<br>3.75 | temperature (° C)<br>fermentation time (h) at 29° C |
| | | Dough: |
| 25.5-26.5<br>5<br>10<br>526<br>100± 1mm<br>16<br>1 | 25.5-26.5<br>9<br>10<br>526<br>100± 1mm<br>16<br>1 | Temperature (° C)<br>Mixing time (min) with med. speed<br>Floor time (min)<br>Scaling weight (g)<br>Average proof height<br>Baking time (min) at about 230° C<br>Cooling time at room temperature (h) |

The baking test results are given in Tables 9-12.

Crumb softness (given in the tables) has been defined using the AACC standard method 74-09 (force required to compress two slices of bread (25 mm) with a 36 mm diameter flat disk plunger by 6.2 mm (25%) at a compression rate of 100 mm/min); the smaller the value, the softer the product.

Table 9

| Bread qualities | Max score | Basic dough | 0.5 % "GMS-90" * | 1 % C | 1 % D |
|---|---|---|---|---|---|
| External qualities: | 30 | | | | |
| Volume | 10 | 8.5 | 9 | 9.25 | 9.5 |
| Symmetry | 5 | 4.75 | 4.5 | 4.5 | 4.25 |
| Crust colour | 10 | 8 | 8 | 8 | 8 |
| "Break & Shred" | 5 | 4.75 | 4.5 | 4.5 | 4.25 |
| Internal qualities: | 70 | | | | |
| Grain | 10 | 8 | 8 | 8 | 8 |
| Texture | 15 | 13.25 | 13.25 | 13.25 | 13 |
| Colour | 10 | 9 | 9 | 9 | 9 |
| Aroma | 10 | 9 | 9 | 9 | 9 |
| Taste | 15 | 13 | 13 | 13 | 13 |
| Mouth feel | 10 | 9 | 9 | 9 | 9 |
| Total score | 100 | 87.25 | 87.25 | 87.5 | 87 |
| Proof height (mm) | | 100 | 99.3 | 100.3 | 100.3 |
| Proof time (min) | | 61 | 60 | 65 | 58 |
| Specific volume ($cm^3/g$) | | 5.50 | 5.60 | 5.65 | 5.70 |
| Crumb softness (3 days) | | 318 ± 6 | 274 ± 5 | 254 ± 5 | 288 ± 6 |
| Dough consistency | | | | more relaxed at moulder** | slighly softer at mixer |

* crumb softener, manuf. Breddo, USA, contains 21 % of monoglycerides, whereby 0.5 % GMS-90 is equivalent to an addition of 1.05 g of monoglycerides per kg of flour

** see Table 2

Table 10

| Bread qualities | Max score | 0.3% Arkady(RKD)* 0.5% GMS-90** | | No Arkady(RKD) 0.5% GMS-90** | | No Arkady(RKD) No GMS-90 1 % C | | No Arkady(RKD) No GMS-90 1 % D | |
|---|---|---|---|---|---|---|---|---|---|
| | | Control | 20 sec vibration | Control | 20 sec vibration | Control | 20 sec vibration | Control | 20 sec vibration |
| External qualities: | 30 | | | | | | | | |
| Volume | 10 | 8.75 | 6.5 | 8.5 | 1.25 | 9 | 2.25 | 8.25 | 4 |
| Symmetry | 5 | 4.25 | 3.75 | 4 | 1.5 | 4 | 1.75 | 4 | 2.75 |
| Crust colour | 10 | 8 | 7 | 8 | 5 | 8 | 5 | 8 | 5.5 |
| "Break & Shred" | 5 | 4.25 | 4 | 4 | 1 | 4.25 | 1.5 | 4.25 | 2 |
| Internal qualities: | 70 | | | | | | | | |
| Grain | 10 | 8 | 7.25 | 6 | 5.75 | 6.75 | 5.75 | 6.75 | 6.25 |
| Texture | 15 | 13 | 12 | 11 | 10.5 | 11.5 | 11.25 | 11.5 | 11.5 |
| Colour | 10 | 9 | 8.75 | 8.5 | 8.25 | 8.5 | 8.25 | 8.5 | 8.5 |
| Aroma | 10 | 8.75 | 8.75 | 9 | 9 | 8.75 | 8.75 | 8.75 | 8.5 |
| Taste | 15 | 12.75 | 12.75 | 12.75 | 12.75 | 12.75 | 12.75 | 12.75 | 12.75 |
| Mouth feel | 10 | 9 | 9 | 8.75 | 8.75 | 8.75 | 8.75 | 9 | 8.75 |
| Total score | 100 | 85.75 | 79.75 | 80.5 | 63.75 | 82.25 | 66 | 81.75 | 70.5 |
| Proof height (mm) | | 99.9 | 99.3 | 100.7 | 100.3 | 100.3 | 100.3 | 100 | 100 |
| Proof time (min) | | 56 | | 65 | | 65 | | 64 | |
| Specific volume ($cm^3/g$) | | 5.38 | 4.91 | 5.31 | 3.86 | 5.42 | 4.06 | 5.28 | 4.41 |
| Crumb softness (1 day) | | 171 ± 1 | | 201 ± 3 | | 174 ± 3 | | 167 ± 2 | |
| Crumb softness (5 days) | | 335 ± 4 | | 352 ± 8 | | 349 ± 6 | | 334 ± 4 | |

* equivalent to 8.4 ppm of bromate

** equivalent to 1.05 g of monoglycerides/kg of flour

Table 11

| Bread qualities | Max score | 0.3% Arkady(RKD)* in sponge 0.5 % GMS-90** | | 0.3% Arkady(RKD)* added to dough 0.5 % GMS-90** | | 1 % E No GMS-90 | |
|---|---|---|---|---|---|---|---|
| | | Control | 20 sec vibration | Control | 20 sec vibration | Control | 20 sec vibration |
| External qualities: | 30 | | | | | | |
| Volume | 10 | 9.25 | 7.5 | 9.25 | 4.5 | 9 | 5 |
| Symmetry | 5 | 4.25 | 4 | 4.25 | 3.5 | 4.25 | 3.5 |
| Crust colour | 10 | 8 | 7.5 | 8 | 6.5 | 8 | 6.5 |
| Break & Shred | 5 | 4.5 | 4 | 4.5 | 3.75 | 4.75 | 3.5 |
| Internal qualities: | 70 | | | | | | |
| Grain | 10 | 8 | 7.75 | 7.5 | 7.5 | 7.5 | 7.5 |
| Texture | 15 | 13 | 12.5 | 12.5 | 12.5 | 13 | 12.25 |
| Crumb colour | 10 | 9 | 9 | 9 | 9 | 8.75 | 8.75 |
| Aroma | 10 | 9 | 9 | 8.75 | 8.75 | 9 | 9 |
| Taste | 15 | 13 | 13 | 13 | 13 | 13 | 13 |
| Mouth feel | 10 | 9 | 9 | 9 | 9 | 9 | 9 |
| Total score | 100 | 87 | 83.25 | 85.75 | 78 | 86.25 | 78 |
| Proof height (mm) | | 99.4 | 99.3 | 100 | 100 | 99.9 | 100 |
| Proof time (min) | | 58 | | 62 | | 63 | |
| Specific volume ($cm^3 \cdot g$) | | 5.49 | 5.10 | 5.47 | 4.51 | 5.41 | 4.63 |

* equivalent to 8.4 ppm of bromate

** equivalent to 1.05 g of monoglycerides/kg of flour

Table 12

| Crumb softness | | | | |
|---|---|---|---|---|
| Formulation | | Combination of to the invention | Bread age | |
| Arkady (RKD) % | GMS-90 % | | 1 day | 5 days |
| 0.3* | 0.5** | - | 171±1 | 335±4 |
| - | 0.5** | - | 201±3 | 352±8 |
| - | - | 134 | 174±3 | 349±6 |
| - | - | 136 | 167±2 | 334±4 |
| 0.3* | 0.5** | - | 147±4 | 294±5 |
| 0.3[a]* | 0.5** | - | 145±4 | 293±7 |
| - | - | 143 | 146±3 | 308±5 |

[a] bromate added to dough instead of sponge

* equivalent to 8.4 ppm of bromate

** equivalent to 1.05 g of monoglycerides per kg of flour

Table 9 gives results from baking tests on the replacement of an emulsifier (monoglycerides) with the combination of the invention. Monoglycerides (bread softener GMS-90) or the combination C and D of the invention were added to the basic dough (containing bromate (Arkady (RKD)) added in the sponge). It appears from the table that the enzyme composition of the invention in combination with lecithin can replace monoglycerides used as emulsifier (cf. the total score obtained by the breads). The loaf volume increased slightly when using the combination of the invention as compared with bread made from the basic dough alone, and the other properties were substantially of the same order. In addition, the use of the

combination of the invention gave slightly softer bread as compared with bread made from the basic dough with the addition of monoglycerides.

Table 10 shows results from baking tests carried out for studying the replacement of bromate with the combinations C and D of the invention. The first dough contained 8.4 ppm of bromate (0.3% Arkady (RKD)) added to the sponge, and 0.11% of monoglycerides (0.5% GMS-90). The second dough contained 0.11% of monoglycerides (0.5% GMS-90) but no bromate. The third dough contained the combination C of the invention without bromate and monoglycerides. Finally, the fourth dough contained the combination D of the invention, similarly without bromate and monoglycerides. In addition, each dough underwent a vibration test of 20 seconds for the assessment of the strength of the dough.

When the combination C of the invention was used, the loaf volume obtained was as good as that obtained with the control containing bromate. No major deficiencies were observed in the external properties of the loaf when bromate was omitted. The proof time, however, was slightly longer with doughs prepared without bromate. As to the test results from the vibration test, the combination D in particular was able to eliminate the negative effects of vibration.

Table 11 shows the results from baking tests carried out for studying the replacement of bromate with the combination E of the invention. Three doughs were prepared of which the first dough contained 8.4 ppm of bromate (0.3 Arkady (RKD)) added to the sponge, and 0.11% of monoglycerides (0.5% GMS-90); the second dough contained 8.4 ppm of bromate (0.3% Arkady (RKD)) added to the dough, and 0.11% of monoglycerides (0.5% GMS-90); and the third dough contained the combination E of the invention without bromate and monoglycerides. It appears from the results that the dough prepared by means of the combination of the invention behaved substantially similarly as the control dough, in which the bromate had been added to the dough instead of the sponge.

Table 12 shows the results from baking tests carried out for comparing the effect of monoglycerides (possibly in combination with bromate) and that of the combinations C, D and E of the invention on the crumb softness when the product was stored. It appears from the results that the combinations C and D of the invention affected the crumb softness as favourably as monoglycerides conventionally used for the purpose (with five days old loaves). The stage at which bromate was added did not affect the ageing of the bread. Bread made with the combination E of the invention was slightly softer than the control after storage for five days.

Example 7

A test bake was carried out for studying further the effects of a simultaneous addition of the enzyme composition optimized for baking purposes and lecithin on white baking. Previous tests have not shown that the use of this enzyme composition could increase the process resistance and loaf volume and improve the anti-staling properties of the product, for instance. It was the object of the test to find out whether lecithin in combination with the enzyme composition could further improve the baking properties of white dough so that qualitatively better bakery products could be obtained.

The product to be baked was a white roll. The qualitative properties of the white flour used in the bake were as follows:

| | |
|---|---|
| Protein content | 10.9% (d.s.) |
| Ash content | 0.79% |
| Falling number | 292 |
| Amylogram | 230 B.U./79° C |
| Add. of ascorbic acid | 15 ppm |

The test bake was carried out with the following formula and process parametres:

Basic formula:

| | |
|---|---|
| White flour | 1,000 g |
| Yeast | 35 g |
| Salt | 20 g |
| Sugar | 20 g |
| Water | 620 g |
| Total | 1,695 g |

Process :

| | |
|---|---|
| Dough mixing | 5 min (Kemper spiral mixer, speed 2) |
| Dough temperature | 27° C |
| Scaling and first moulding | 60 g (Rekord teiler) |
| First proof time | 10 min |
| Proofing | 40 min/75% rH, 35° C |
| Baking time | 18 min/220° C |

The following doughs were prepared: (1) a basic dough with the basic formula, (2) a basic dough with the addition of lecithin, (3) a basic dough with the enzyme addition according to the invention, and (4) a basic dough with the addition of lecithin and enzyme.

The added lecithin and enzyme amounts in doughs (2), (3) and (4) were as follows:

| | (2) | (3) | (4) |
|---|---|---|---|
| GO | - | 1 mg/kg | 1 mg/kg |
| Cell..hemicell: | - | 15 " | 15 " |
| Fungal protease ("Fungal protease", manuf. Biocon, Ireland) | - | 45 " | 45 " |
| Fungal alpha-amylase ("Sal-Conc. 90 000", manuf. Shin Nihon, Japan) | - | 5 " | 5 " |
| Lecithin. Emulpur N | 0.4% | - | 0.4% |

The amounts of added enzymes is given in mg per kg of flour and the amount of added lecithin in % on flour.

The added amount of cellulolytic and hemicellulolytic enzymes and glucose oxidase as enzyme activities per kg of flour were as follows:

| | Added enzyme activities U/kg | | | |
|---|---|---|---|---|
| | Xyl. | FP | CMC | GO |
| Flours (3) and (4) | 405 | 5.7 | 140 | 105 |

The results are shown in the following table:

| Product properties | Baking series | | | |
|---|---|---|---|---|
| | (1) | (2) | (3) | (4) |
| Weight (g) | 45 | 45 | 48 | 47 |
| Height (mm) | 46 | 48 | 50 | 48 |
| Width (mm) | 77 | 82 | 78 | 80 |
| Volume (ml/prod.) | 153 | 164 | 177 | 182 |
| Specific volume ($cm^3/g$) | 3.4 | 3.6 | 3.7 | 3.9 |
| Sensory evaluation (texture/crumb properties) | satisf. | good | good | excellent |

The results show that the mere addition of lecithin or an enzyme composition useful in baking improves the roll baking result. Both additions increase the roll volume by 5-10% in average. As to the crumb texture, making differences can be found between products prepared with an addition of lecithin and enzymes, respectively. Lecithin gives a more even grain structure with smaller pores as compared with the enzyme composition. The addition of lecithin gives the dough a rather slack, slightly sticky texture, whereas the enzyme mixture strengthens the dough giving good handling properties. Simultaneous use of lecithin and the enzyme composition in baking clearly affects favourably the baking properties. The elastic dough has improved handling and process properties. The grain structure of the final product is more uniform and softer as compared with products prepared with a mere addition of lecithin or enzymes. In addition, the external properties of the product are more even (crust texture). The simultaneous use of lecithin and the enzyme composition simultaneously increases the bread volume by about 15% as compared with a product prepared without any additions.

Preliminary experiments have demonstrated that the enzyme composition of the invention with or without lecithin works also in doughs where higher amounts of fat and/or sugar and/or spices are present, such as in doughs for sweet goods, like cakes.

The effective amount of cellulose and hemicellulose degrading enzymes is mutually dependent on the level of each other. The levels are also dependent on the microbial source used in enzyme production. Furthermore, the effective amount of cellulose and hemicellulose (specified as xylen) degrading enzymes is dependent on the levels of other hemicellulose degrading enzyme activities.

Foregoing general discussion and experimental examples are intended to be illustrative of the present invention, and are not to be considered as limiting. Other variations within the spirit and scope of this invention are possible and will present themselves to those skilled in the art.

## Claims

1. A method of improving the properties of dough and the quality of the baked product, **characterized** by adding to the dough, dough ingredients, ingredient mixture or dough additives or additive mixture an enzyme preparation comprising hemicellulose and/or cellulose degrading enzymes and glucose oxidase, or sulphydryl oxidase and glucose oxidase.

2. A method according to claim 1, **characterized** in that the enzyme preparation is added in an amount of about 0-50,000 units of hemicellulase; about 0-50,000 units of cellulase; about 5-2,500 units of glucose oxidase; and about 0-800 units of sulphydryl oxidase, calculated per kg of flour.

3. A method according to claim 2, **characterized** in that the enzyme preparation is added in an amount of 10-10,000 units of hemicellulase; 10-10,000 units of cellulase; 35-1,000 units of glucose oxidase; and 0-300 units of sulphydryl oxidase, calculated per kg of flour.

4. A method according to claim 1, **characterized** in that the dough is prepared using a straight dough process, a sour dough process, the Chorleywood Bread Process or the Sponge and Dough process.

5. A method according to claim 1, **characterized** in that the baked product is bread.

6. A method according to claim 1, **characterized** in that the baked products are sweet goods.

7. A method according to any of the preceding claims, **characterized** in that the dough additive or additive mixture contains lecithin.

8. A method according to claim 6, **characterized** in that lecithin is used in an amount of 0.1-1.4%, preferably 0.2-0.8%, specified as 100% lecithin, calculated on the flour.

9. An enzyme preparation useful in baking, **characterized** in that it comprises hemicellulose and/or cellulose degrading enzymes and glucose oxidase, or glucose oxidase and sulphydryl oxidase.

10. A pre-mixture useful in baking, **characterized** in that it comprises hemicellulose and/or cellulose degrading enzymes and glucose oxidase, or glucose oxidase and sulphydryl oxidase, as mixed with a carrier.

11. A pre-mixture according to claim 8, **characterized** in that the carrier is flour, dry milk, sugar, fat or their mixture or a baking additive or additive mixture.

12. A pre-mixture according to claim 9, **characterized** in that the carrier is a baking additive or additive mixture containing lecithin.

European Patent Office

# EUROPEAN SEARCH REPORT

Application Number

EP 89 10 6729

## DOCUMENTS CONSIDERED TO BE RELEVANT

| Category | Citation of document with indication, where appropriate, of relevant passages | Relevant to claim | CLASSIFICATION OF THE APPLICATION (Int. Cl.4) |
|---|---|---|---|
| D,X | US-A-2 783 150 (H.G. LUTHER) * Column 1, line 40 - column 2, line 11; column 2, line 45 - column 3, line 5; claim 1 * | 1-6,9-11 | A 21 D 8/04 |
| A | GB-A-1 216 556 (DELMAR CHEMICALS) * Page 1, line 60 - page 3, line 26; examples 1-5; claims 1-7,9-17 * | 1-6,9-11 | |
| A | EP-A-0 132 289 (KYOWA HAKKO KOGYO CO., LTD) * Page 1, line 25 - page 2, line 11; page 3, lines 1-33; examples 1-5; claims 1,4,6 * | 1-5,9-11 | |
| A | CHEMICAL ABSTRACTS, vol. 71, no. 11, 24th November 1969, pages 225-226, abstract no. 100605k, Columbus, Ohio, US; M.V. POLYAK et al.: "Glucose oxidase as a conditioner in bread baking", & FERMENTY MED., PISHCH. PROM. SEL. KHOZ. 1968, 155-7 | 1-5,9-11 | |

TECHNICAL FIELDS SEARCHED (Int. Cl.4)

A 21 D

The present search report has been drawn up for all claims

| Place of search | Date of completion of the search | Examiner |
|---|---|---|
| THE HAGUE | 09-06-1989 | GROENENDIJK M.S.M. |

CATEGORY OF CITED DOCUMENTS

X : particularly relevant if taken alone
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